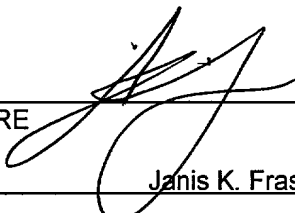


SUBSTITUTE FORM PTO-1390		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 00786-407001
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (IF KNOWN) 09/647019
INTERNATIONAL APPLICATION NO. PCT/AU99/00220	INTERNATIONAL FILING DATE March 26, 1999	PRIORITY DATE CLAIMED March 27, 1998	
TITLE OF INVENTION NOVEL MOLECULES EXPRESSED DURING MUSCLE DEVELOPMENT AND GENETIC SEQUENCES ENCODING THE SAME			
APPLICANT(S) FOR DO/EO/US Richard P. Harvey, Nadia A. Rosenthal, Stephen J. Palmer and Antonio Musaro			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> A translation of the International Application (35 U.S.C. 371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). <i>Items 11. to 16. below concern other documents or information included:</i> 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of power of attorney and/or address letter. 16. <input type="checkbox"/> Other items or information: <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>			
'Express Mail' mailing label number EL228026666US Date of Deposit September 26, 2000 I hereby certify that this paper or fee is being deposited with the United States Postal Service 'Express Mail Post Office to Addressee' service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner For Patents, Washington, D.C. 20231 <u>Samantha Bell</u> <u>Samantha Bell</u>			

430 Rec'd PCT/PTO 26 SEP 2000

U.S. APPLICATION NO. (IF KNOWN) 09/647019		INTERNATIONAL APPLICATION NO. PCT/AU99/00220		ATTORNEY'S DOCKET NUMBER 00786-407001	
17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):					
Search report has been prepared by the EPO or JPO..... \$840				\$0.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) .. \$670				\$0.00	
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).... \$690				\$0.00	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$970				\$970.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4) \$96				\$0.00	
ENTER APPROPRIATE BASIC FEE AMOUNT				\$970.00	
Surcharge of \$130 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).				\$0.00	
Claims	Number Filed	Number Extra	Rate		
Total Claims	65 - 20	45	x \$18	\$810.00	
Independent Claims	18 - 3	15	x \$78	\$1,170.00	
Multiple Dependent Claims(s) (if applicable)			+ \$260	\$0.00	
TOTAL OF ABOVE CALCULATIONS				\$1,980.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28.)				\$0.00	
SUBTOTAL				\$2,950.00	
Processing fee of \$130 for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(f))				\$0.00	
TOTAL NATIONAL FEE				\$2,950.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31).				\$0.00	
TOTAL FEES ENCLOSED				\$2,950.00	
				Amount to be refunded	
				Charged	
a. <input checked="" type="checkbox"/> A check in the amount of \$2,950.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 06-1050 in the amount of \$0.00 to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1050. A duplicate copy of this sheet is enclosed.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
Janis K. Fraser, Ph.D., J.D. FISH & RICHARDSON P.C. 225 Franklin Street Boston, MA 02110-2804 (617) 542-5070 phone (617) 542-8906 facsimile			<div style="text-align: right;"> <i>Anita L. Meiklejohn</i> Reg. No. 35,283 </div> <div style="text-align: center;">  SIGNATURE </div> <div style="text-align: center;"> NAME Janis K. Fraser, Ph.D., J.D. </div> <div style="text-align: right;"> 34,819 REGISTRATION NUMBER </div>		

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Richard P. Harvey et al. Art Unit : Unknown
Serial No. : Examiner : Unknown
Filed : HEREWITH
Title : NOVEL MOLECULES EXPRESSED DURING MUSCLE DEVELOPMENT
AND GENETIC SEQUENCES ENCODING THE SAME

Box PCT

Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

In the Claims:

In claim 5, line 1, delete "or 3".
In claim 10, line 1, delete "or 8".
In claim 14, line 1, delete "or 13".
In claim 17, line 1, delete "or 16".
In claim 18, line 1, delete "or 17".
In claim 21, line 1, delete "or 20".
In claim 22, line 1, delete "or 20".
In claim 24, line 1, delete ", 21, 22 or 23".
In claim 26, line 1, delete "any one of claims 15-25" and insert --claim 15--.
In claim 27, line 1, delete "any one of claims 15-25" and insert --claim 15--.
In claim 31, line 3, delete "any one of claims 15-25" and insert --claim 15--.

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL228026666US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

Date of Deposit September 26, 2000

Signature

Samantha Bell
Samantha Bell

Typed or Printed Name of Person Signing Certificate

097847019

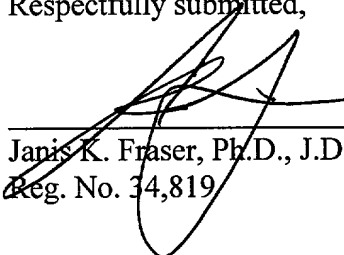
In claim 34, line 3, delete "any one of claims 1-14" and insert --claim 1--.
In claim 37, line 3, delete "any one of claim 15-25" and insert --claim 15--.
In claim 39, line 1, delete "or 38".
In claim 40, line 3, delete "any one of claims 1-14" and insert --claim 1--.
In claim 42, line 1, delete "or 41".
In claim 45, line 1, delete "or 44".
In claim 48, line 2, delete "any one of claims 15-25" and insert --claim 15--.
In claim 49, lines 2-3, delete "any one of claims 1-14" and insert --claim 1--.
In claim 50, line 1, delete "any one of claims 46 to 49" and insert --claim 46--.
In claim 51, line 1, delete "any one of claims 46 to 50" and insert --claim 46--.
In claim 59, line 1, delete "any one of claims 15-25" and insert --claim 15--.
In claim 60, lines 1-2, delete "any one of claims 1-14" and insert --claim 1--.
In claim 61, line 1, delete "or 60".
In claim 62, line 1, delete "or 60".

REMARKS

Claims 1 to 65 are pending in this application, claims 5, 10, 14, 17, 18, 21, 22, 24, 26, 27, 31, 34, 37, 39, 40, 42, 45, 48-51, and 59-62 having been amended to delete multiple dependency. No new matter has been added. Please apply any other charges or credits to Deposit Account No. 06-1050.

Date: 26 Sept 2000

Respectfully submitted,


Janis K. Fraser, Ph.D., J.D.
Reg. No. 34,819

Anita L. Meiklejohn
Reg. No.
35,283

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110-2804
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

13 JUN 2001

#-7

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Richard P. Harvey et al. Art Unit : Unknown
 Serial No. : 09/647,019 Examiner : Unknown
 Filed : September 26, 2000
 Title : NOVEL MOLECULES EXPRESSED DURING MUSCLE DEVELOPMENT
 AND GENETIC SEQUENCES ENCODING THE SAME

BOX PCT

Commissioner for Patents
 Washington, D.C. 20231

RESPONSE TO NOTIFICATION TO COMPLY WITH REQUIREMENTS
 FOR PATENT APPLICATIONS CONTAINING
 NUCLEOTIDE AND/OR AMINO ACID SEQUENCES

In response to the communication dated April 13, 2001 (copy enclosed), applicant submits herewith a Sequence Listing in computer readable form as required by 37 CFR §1.824. In addition, applicant submits a substitute Sequence Listing as required under 37 CFR §1.823(a) and a statement under 37 CFR §1.821(f).

Applicant respectfully requests entry of the paper copy and computer readable copy of the Sequence Listing filed herewith for the instant application. Furthermore, applicant requests entry of the following amendments.

In the specification:

Replace the original Sequence Listing with the substitute Sequence Listing filed herewith.

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL259011485US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

June 13, 2001

Date of Deposit

Signature

Typed or Printed Name of Person Signing Certificate

Joshua Crovin
 Joshua Crovin

09/647,019

Replace the paragraph beginning at page 13, line 21, with the following rewritten paragraph:

--**Figure 3** is a schematic representation of the predicted amino acid sequence of human (SEQ ID NO:4), mouse (SEQ ID NO:2), and Xenopus (SEQ ID NO:5) Csl protein. The sequences have been aligned using DNASTar software.--

Replace the paragraph beginning at page 15, line 23, with the following rewritten paragraph:

--**Figure 12** is schematic representation of the alignment of EF-Hand protein binding sites to Incomplete IQ (SEQ ID NOs:11 to 15, including the consensus sequence) and Non-IQ (SEQ ID NOs:16 to 20) sites in various proteins. Proposed modalities of binding are also represented diagrammatically.--

09/26/00 16:04:50

REMARKS

Applicant hereby submits that the enclosures fulfill the requirements under 37 C.F.R. § 1.821-1.825. The amendments in the specification merely replace the original Sequence Listing with an amended Sequence Listing and insert sequence identifiers in the specification. The substitute Sequence Listing includes the amino acid sequences disclosed in Figures 3 and 12 that were inadvertently omitted from the original Sequence Listing. I hereby state, as required by 37 C.F.R. §1.821(g), that the enclosed submission includes no new matter.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment.

Please apply any charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date:

June 13, 2001



Janis K. Fraser, Ph.D., J.D.
Reg. No. 34,819

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110-2804
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

“Version With Markings to Show Changes Made”

In the specification:

Paragraph beginning at page 13, line 21, has been amended as follows:

Figure 3 is a schematic representation of the predicted amino acid sequence of human (SEQ ID NO:4), mouse (SEQ ID NO:2), and Xenopus (SEQ ID NO:5) Csl protein. The sequenecs have been aligned using DNASTar software.

Paragraph beginning at page 15, line 23, has been amended as follows:

Figure 12 is schematic representation of the alignment of EF-Hand protein binding sites to Incomplete IQ (SEQ ID NOs:11 to 15, including the consensus sequence) and Non-IQ (SEQ ID NOs:16 to 20) sites in various proteins. Proposed modalities of binding are also represented diagrammatically.

09/647,019-043604

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Richard P. Harvey et al. Art Unit : Unknown
 Serial No. : 09/647,019 Examiner : Unknown
 Filed : September 26, 2000
 Title : NOVEL MOLECULES EXPRESSED DURING MUSCLE DEVELOPMENT
 AND GENETIC SEQUENCES ENCODING THE SAME

BOX PCT

Commissioner for Patents
 Washington, D.C. 20231

VERIFIED STATEMENT UNDER 37 CFR §1.821(f)

I, Katica Magovcevic, declare that I personally prepared the paper and the computer-readable copy of the Sequence Listing filed herewith for the above-identified application and that the content of both is the same.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of The United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 5/30/01

Katica Magovcevic
 Katica Magovcevic

Fish & Richardson P.C.
 225 Franklin Street
 Boston, MA 02110-2804
 (617) 542-5070 telephone
 (617) 542-8906 facsimile

20268638.doc

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I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

June 13, 2001
 Date of Deposit

Joshua Cronin
 Signature
 Joshua Cronin
 Typed or Printed Name of Person Signing Certificate

37/p85

09/647019

430 Rec'd PCT/PTO 26 SEP 2000

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NOVEL MOLECULES EXPRESSED DURING MUSCLE DEVELOPMENT AND GENETIC SEQUENCES ENCODING THE SAME**FIELD OF THE INVENTION**

5 The present invention relates to novel molecules expressed during muscle development and to genetic sequences encoding same. More particularly, the present invention relates to novel molecules capable of, *inter alia*, modulating heart and skeletal muscle cell functional activity and to genetic sequences encoding same. Even more particularly, the present invention provides a novel molecule referred to herein as "Csl" and to genetic sequences encoding same. The molecules of the present invention are useful, for example, in therapy, diagnosis and as a screening tool for therapeutic agents capable of modulating muscle cell functional activity.

BACKGROUND OF THE INVENTION

15

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

The genetic basis of skeletal muscle development and differentiation has received much attention over the last two decades, especially with regard to the role of the homeobox genes, the basic helix-loop-helix myogenic factors and members of the myocyte enhancer binding factor family (1).

The genetic basis of heart development has enjoyed less attention. This is, in part, due to work on the role of the NK-2 class of homeobox genes. The drosophila NK-2 class homeobox gene *tinman* is so named because flies without this gene, apart from other defects, lack the muscular dorsal vessel that can be loosely described as a heart (2). The search for mammalian homologs of NK-2 revealed several genes amongst which the gene Nkx2.5 was found to be expressed in the developing mouse heart (3). Mouse embryos homozygous for a null mutation of Nkx2.5 (Nkx2.5-/-) form a linear heart tube but fail to

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undergo the morphogenetic process of heart looping during the early stages of cardiogenesis and the embryos die due to hemodynamic insufficiency (4). The cells of the unlooped heart tube show several characteristics of normal cardiocytes such as the expression of many myofilament genes but the knockouts demonstrate that Nkx2.5 is
5 clearly a major determinant of cardiac development.

Already some genes have been identified that fail to be activated in Nkx2.5^{-/-} hearts including the myosin light chain 2V (MLC2V) (4) and the atrial natriuretic factor ANF, which is known to have an Nkx2.5 response element (5). An ankyrin repeat-containing
10 nuclear protein CARP is also down regulated in the Nkx2.5^{-/-} model. MLC2v may be regulated directly by CARP and its binding partner YB-1 (6).

In work leading up to the present invention, the inventors have identified a novel gene, designated herein "*Csl*". In accordance with the present invention, *Csl* mRNA is
15 detectable in the mouse heart from about 8 days post-coitum ("dpc") through to adulthood but is absent from mice carrying a Nkx2.5^{-/-} mutation. It is also expressed in skeletal muscle. The identification of this new gene permits rational design of drugs for modulation of *Csl* activity and further identification of a range of molecules for use in therapy, diagnosis, antibody generation and modulation of muscle cell development.
20 These molecules include the product of the *Csl* gene, *Csl*. Other molecules contemplated herein may act as either agonists or antagonists of *Csl*'s functions and will be useful *inter alia* in attenuation of muscle frailty in aging, treatment of muscular and myotonic dystrophies, the prevention of cardiomyopathy and the regulation of myogenic differentiation.

25

SUMMARY OF THE INVENTION

Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the Examples. A summary of the
30 sequences with given SEQ ID NOs is provided before the Examples.

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Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

5

One aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence corresponding to a gene which is expressed in heart muscle from about 8 dpc in murine species or its equivalents in other mammalia species such as humans.

10

Another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding, or a nucleotide sequence complementary to a nucleotide sequence encoding, an amino acid sequence substantially as set forth in SEQ ID NO:2 or a derivative or homolog or mimetic thereof or having at least about 45% or greater
15 similarity to at least 20 contiguous amino acids in SEQ ID NO:2 or a derivative or homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.

Still another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding, or a nucleotide sequence complementary to a nucleotide
20 sequence, encoding an amino acid sequence substantially as set forth in SEQ ID NO:4 or a derivative, homolog or mimetic thereof or having at least about 45% or greater similarity to at least 20 contiguous amino acids in SEQ ID NO:4 or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.

25 Still yet another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding, or a nucleotide sequence complementary to a nucleotide sequence encoding, an amino acid sequence substantially as set forth in SEQ ID NO:5 or a derivative, homolog or mimetic thereof or having at least about 45% or greater similarity to at least 20 contiguous amino acids in SEQ ID NO:5 or a derivative, homolog,
30 analog, chemical equivalent or mimetic of said nucleotide sequence.

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A further aspect of the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in SEQ ID NO:1 or a derivative, homolog or mimetic thereof capable of hybridising to SEQ ID NO:1 under low stringency conditions at 42°C.

5

Still another further aspect of the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in SEQ ID NO:3 or a derivative, homolog or mimetic thereof capable of hybridising to SEQ ID NO:3 under low stringency conditions at 42°C.

10

Still yet another further aspect of the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in SEQ ID NO:1 or a derivative, homolog or mimetic thereof capable of hybridising to SEQ ID NO:1 under low stringency conditions at 42°C and which encodes an amino acid sequence corresponding to
15 an amino acid sequence set forth in SEQ ID NO:2 or a sequence having at least about 45% similarity to at least 20 contiguous amino acids in SEQ ID NO:2.

Even yet another further aspect of the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in SEQ ID NO:3 or a
20 derivative, homolog or mimetic thereof capable of hybridising to SEQ ID NO:3 under low stringency conditions at 42°C and which encodes an amino acid sequence corresponding to an amino acid sequence set forth in SEQ ID NO:4 or a sequence having at least about 45% similarity to at least 20 contiguous amino acids in SEQ ID NO:4.

25 In another aspect the present invention contemplates a nucleic acid molecule comprising a sequence of nucleotides substantially as set forth in SEQ ID NO:1 or 3.

In yet another aspect of the present invention contemplates a genomic nucleic acid molecule or a derivative or homolog or mimetic thereof capable of hybridising to SEQ ID
30 NO:1 or 3 or a derivative or homolog or mimetic thereof under low stringency conditions

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at 42°C.

In another aspect the present invention provides a nucleotide sequence corresponding to *Csl* is a cDNA sequence comprising a sequence of nucleotides as set forth in SEQ ID NO:3 or is a derivative, homolog, analog, chemical equivalent or mimetic thereof including a nucleotide sequence having similarity to SEQ ID NO:3.

In yet another aspect the present invention provides a nucleotide sequence corresponding to *Csl* is a genomic nucleotide sequence or a derivative, homolog or mimetic thereof corresponding to a gene map as set forth in Figure 1.

In still yet another aspect the present invention provides a nucleotide sequence corresponding to *Csl* is a genomic nucleotide sequence or a derivative, homolog or mimetic thereof corresponding to a gene map as set forth in Figure 2.

In a further aspect the nucleotide sequence corresponding to *Csl* is a genomic nucleotide sequence or a derivative, homolog or mimetic thereof having exon regions of which 5 comprise:

- 20 Exon 1 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:6;
- Exon 2 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:7;
- Exon 3 comprising a nucleotide sequence corresponding to the nucleotide sequence
- 25 substantially as set forth in SEQ ID NO:8;
- Exon 4 comprising the nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:9; and
- Exon 5 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:10;

30

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or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.

In another further aspect the present invention is directed to an isolated nucleic acid molecule encoding *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleic acid molecule, said nucleic acid molecule selected from the list consisting of:

- 10 (i) A nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence set forth in one or more of SEQ ID NO:2 or 4 or 5 or a derivative or homolog or mimetic thereof or having at least about 45% similarity to at least 20 contiguous amino acids in one or more of SEQ ID NO: 2 or 4 or 5 or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.
- 15 (ii) A nucleic acid molecule comprising a nucleotide sequence substantially as set forth in one or more of SEQ ID NO:1 or 3 or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.
- 20 (iii) A nucleic acid molecule comprising a nucleotide sequence corresponding to a gene map as set forth in Figures 1 or 2 or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.
- (iv) A nucleic acid molecule having exon regions of which 5 comprise:
- 25 Exon 1 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:6;
Exon 2 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:7;
Exon 3 comprising a nucleotide sequence corresponding to the nucleotide
30 sequence substantially as set forth in SEQ ID NO:8;

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Exon 4 comprising the nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:9; and

Exon 5 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:10,

5

or a derivative, homolog, analog, chemical equivalent or mimetic thereof.

(v) A nucleic acid molecule capable of hybridising under low stringency conditions at 42°C to the nucleotide sequence substantially as set forth in one or more of SEQ ID NO:1 or 3 or a derivative or homolog or mimetic thereof and encoding an amino acid sequence corresponding to an amino acid sequence as set forth in one or more of SEQ ID NO:2 or 4, respectively, or a derivative or homolog or mimetic thereof or a sequence having at least about 45% similarity to at least 20 contiguous amino acids in one or more of SEQ ID NO:2 or 4, respectively, or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleic acid molecule.

(vi) A nucleic acid molecule capable of hybridising to the nucleic acid molecule of paragraphs (i), (ii) or (v) under low stringency conditions at 42°C and encoding an amino acid sequence having at least about 45% similarity to at least 20 contiguous amino acids in one or more of SEQ ID NO:2 or 4.

(vii) A nucleic acid molecule capable of hybridising to the nucleic acid molecule of paragraphs (iii) or (iv) under low stringency conditions at 42°C.

25

(viii) A derivative, homolog, analog or chemical equivalent or mimetic of the nucleic acid molecule of paragraphs (i) or (ii) or (iii) or (iv) or (v) or (vi) or (vii).

Another aspect of the present invention is directed to an isolated protein selected from the list consisting of:

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- 5 (i) A protein having an amino acid sequence substantially as set forth in one or more of SEQ ID NO:2 or 4 or 5 or derivative, homolog, analog, chemical equivalent or mimetic thereof or a sequence having at least about 45% similarity to at least 20 contiguous amino acids in one or more of SEQ ID NO:2 or 4 or 5 or a derivative, homolog or mimetic thereof.
- 10 (ii) A protein encoded by a nucleotide sequence substantially as set forth in one or more of SEQ ID NO:1 or 3 or a derivative, homolog or mimetic thereof or a sequence encoding an amino acid sequence having at least about 45% similarity to at least 20 contiguous amino acids in one or more of SEQ ID NO:2 or 4, or a derivative, homolog or mimetic thereof.
- 15 (iii) A protein encoded by a nucleotide sequence corresponding to a gene map substantially as set forth in Figure 1 or 2 or a derivative, homolog or mimetic thereof.
- (iv) A protein encoded by a genomic *Csl* nucleotide sequence having exon regions of which 5 comprise:
- 20 Exon 1 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:6;
Exon 2 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:7;
Exon 3 comprising a nucleotide sequence corresponding to the nucleotide
25 sequence substantially as set forth in SEQ ID NO:8;
Exon 4 comprising the nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:9; and
Exon 5 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:10,
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or a derivative, homolog, analog, chemical equivalent or mimetic thereof.

- (v) A protein encoded by a nucleic acid molecule capable of hybridising to the nucleotide sequence as set forth in one of SEQ ID NO:1 or 3 or a derivative or homolog or mimetic thereof under low stringency conditions at 42°C and which encodes an amino acid sequence substantially as set forth in SEQ ID NO:2 or 4, respectively, or a derivative or homolog or mimetic thereof or an amino acid sequence having at least about 45% similarity to at least 20 contiguous amino acids in one or more SEQ ID NO:2 or 4.
- (vi) A protein as defined in paragraphs (i) or (ii) or (iii) or (iv) or (v) in a homodimeric form.
- (vii) A protein as defined in paragraphs (i) or (ii) or (iii) or (iv) or (v) in heterodimeric form.

A further aspect of the present invention contemplates a method for modulating expression of *Csl* in a mammal, said method comprising contacting the *Csl* gene with an effective amount of an agent for a time and under conditions sufficient to up-regulate or down-regulate or otherwise modulate expression of *Csl*.

Another aspect of the present invention contemplates a method for modulating activity of *Csl* in a mammal, said method comprising administering to said mammal a modulating effective amount of an agent for a time and under conditions sufficient to increase or decrease *Csl* activity.

Still another aspect of the present invention contemplates a method of modulating muscle cell functional activity in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a nucleotide sequence encoding *Csl* or sufficient to modulate

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the activity of Csl.

Yet another aspect of the present invention contemplates a method of modulating muscle cell functional activity in a mammal said method comprising administering to said
5 mammal an effective amount of Csl or *Csl*.

Still yet another aspect of the present invention is directed to a method of modulating muscle cell functional activity in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to
10 modulate the expression of a nucleotide sequence encoding *Csl* or sufficient to modulate the activity of Csl wherein said *Csl* expression product or said Csl interacts, binds or otherwise associates with an EF-Hand target.

A further aspect of the present invention contemplates a method of modulating muscle cell
15 functional activity in a mammal said method comprising administering to said mammal an effective amount of Csl or *Csl* wherein said Csl or the expression product of said *Csl* interacts, binds or otherwise associates with an EF-Hand target.

Another further aspect of the present invention is directed to a method of modulating
20 cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of Csl or *Csl* for a time and under conditions sufficient to modulate the activity of one or more components of a calcineurin-dependent signalling pathway.

25 Still another further aspect of the present invention is directed to a method of modulating cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a nucleotide sequence encoding *Csl* or sufficient to modulate the activity of Csl wherein said *Csl* expression product or Csl modulates the activity of one
30 or more of the components of a calcineurin dependent signalling pathway.

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Another aspect of the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of an agent capable of modulating the expression of *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate muscle cell
5 functional activity.

In yet another aspect the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of an agent capable of modulating the activity of *Csl* or a derivative, homolog, analog, chemical equivalent or
10 mimetic thereof for a time and under conditions sufficient to modulate muscle cell functional activity.

In still yet another aspect the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of *Csl* or a
15 derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate muscle cell functional activity.

Yet another aspect of the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of *Csl* or a
20 derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate muscle cell functional activity.

A further aspect the present invention relates to the use of an agent capable of modulating the expression of *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic
25 thereof in the manufacture of a medicament for the modulation of muscle cell functional activity.

Another further aspect of the present invention relates to the use of an agent capable of modulating the expression of *Csl* or a derivative, homolog, analog, chemical equivalent or
30 mimetic thereof in the manufacture of a medicament for the modulation of muscle cell

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functional activity.

Still another further aspect of the present invention relates to the use of Csl or Csl or a derivative, homolog, analog, chemical equivalent or mimetic thereof in the manufacture of
5 a medicament for the modulation of muscle cell functional activity.

Still yet another further aspect of the present invention relates to agents for use in modulating Csl or a derivative, homolog, analog, chemical equivalent or mimetic thereof wherein modulating said Csl modulates muscle cell functional activity.

10

A further aspect of the present invention relates to agents for use in modulating Csl expression or a derivative, homolog, analog, chemical equivalent or mimetic thereof wherein modulating expression of said Csl modulates muscle cell functional activity.

15 Another aspect of the present invention relates to Csl or Csl or a derivative, homolog, analog, chemical equivalent or mimetic thereof for use in modulating muscle cell functional activity.

In yet another further aspect the present invention contemplates a pharmaceutical
20 composition comprising Csl, Csl or an agent capable of modulating Csl expression or Csl activity or derivative, homolog, analog, chemical equivalent or mimetic thereof together with one or more pharmaceutically acceptable carriers and/or diluents.

Still another aspect of the present invention is directed to antibodies to Csl.

25

Another aspect of the present invention contemplates a method for detecting Csl in a biological sample from a subject said method comprising contacting said biological sample with an antibody specific for Csl or its derivatives or homologs for a time and under conditions sufficient for an antibody-Csl complex to form, and then detecting said
30 complex.

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Yet another aspect of the present invention is directed to a method of diagnosing or monitoring a disease condition in a mammal, which disease condition is characterized by aberrant muscle cell functional activity, said method comprising screening for *Csl* and/or *Csl* in a biological sample isolated from said mammal.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Colour copies of all photographic representations are available upon request.

10 **Figure 1** is a schematic representation of the putative genomic structure of the mouse *Csl* locus. Two lambda genomic DNA clones have been identified which contain exons 1 and 2 and exon 5. Lambda clone 1 has been characterized in detail and the splice junctions of both exons are identical to the human. The splice sites of exons 3, 4 and 5 are extrapolated from the structure of the human gene.

15

Figure 2 is a schematic representations of the structure of the human *Csl* locus. The entire region has been fully sequenced from two overlapping cosmid clones (accession numbers U73509 and U73508). The gene maps to the X chromosome and spans approximately 50 kb.

20

Figure 3 is a schematic representation of the predicted amino acid sequence of human, mouse and *Xenopus* *Csl* protein. The sequences have been aligned using DNASTar software.

25 **Figure 4** is a photographic representation of a ribonuclease protection assay analysing *Csl* expression in a range of adult tissues. Predominant expression is seen in heart and skeletal muscle (Sk. Muscle; Tongue) with minor levels in testes, lung and skull. A cyclophilin probe (Cyclo) was used as a control for RNA integrity.

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Figure 5 is a photographic representation of Northern analysis of Csl expression in heart and different skeletal muscles of wildtype mice, and mice carrying an MLC-IGF-I transgene. Csl transcript of approximately 1 kb is seen at robust levels in all muscle types analysed, including predominantly slow (Soleus) and fast (Gastrocnemeus) muscles. No major increase in expression is seen in the transgenic muscles. Ethidium Bromide (EtBr) staining of the Northern gel is shown to highlight RNA loading and integrity.

Figure 6 is a photographic representation of Northern analysis of Csl expression in hypertrophic hearts of renal artery-banded rats. Lanes 1&2: different whole heart samples from spontaneously hypertensive rats. Lanes 3&4: different whole heart samples from renal artery-banded spontaneously hypertensive rats. Note increased expression of atrial natriuretic factor (Anf) and alpha-skeletal actin mRNAs, markers of cardiac hypertrophy, in banded samples. Csl expression is not significantly altered by the procedure.

Figure 7 is a photographic representation of:

Top panel: Comparison of C2C12, L6E9, L6/Csl, L6MLC/IGF-I and L6/IGF/Csl myocyte morphology using immunofluorescence analysis of myosin (using MF-20 anti-muscle myosin antibody).

20

Bottom panel: Western blot analysis of muscle myosin protein levels (MF-20 antibody) with and without transfected FLAG-Csl in muscle cell lines shown above.

Figure 8 is a photographic representation of:

25

Top panel: Subcellular localisation of calcineurin in L6E9 and L6MLC/IGF-I myocytes, with Hoechst nuclear stain (blue [photographs A and B]) or anti-Calcineurin A antibodies (red [photographs C and D]).

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Bottom panel: Western analysis of Calcineurin A protein levels with and without transfected FLAG-Csl in various muscle cell lines.

Figure 9 is a photographic representation of immunofluorescence analysis of myc epitope-tagged Csl expression in the L6E9 system. Hoechst nuclear staining (blue [photographs A, B and C]) and anti-myc immunofluorescence (red [photographs D, E and F]) in L6E9 or L6MLC/IGF-I cultures transiently transfected with an empty myc-tag vector (two lefthand panels), or with an MLC/myc-Csl vector (right-hand panels), two days after initiation of differentiation. Note predominantly nuclear localisation of small myc epitope-containing peptide expressed from the vector.

Figure 10 is a photographic representation of immunofluorescence analysis of myc epitope-tagged Csl expression in the L6E9 system (as in Figure 9). Hoechst nuclear staining (blue [photographs A and B]) and anti-myc immunofluorescence (red [photographs C and D]) L6MLC/IGF-I cultures transiently transfected with an empty myc-tag vector (left-hand panels), or with an MLC/myc-Csl vector (right-hand panels), five days after initiation of differentiation. Note predominantly nuclear localisation of small myc epitope-containing peptide expressed from the vector.

Figure 11 is a graphical representation of the prediction of the alpha helical content of Csl using the BMERC Protein Sequence Analysis System.

Figure 12 is a schematic representation of the alignment of EF-Hand protein binding sites to Incomplete IQ and Non-IQ sites in various proteins. Proposed modalities of binding are also represented diagrammatically.

Figure 13 is a photographic representation of poly acrylamide gel analysis of bacterial Csl after cleavage from immobilised GST-Csl column using thrombin.

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Figure 14 is a photographic representation of Western blot analysis of bacterial Csl and COS cell-expressed Csl and FLAG-Csl using Csl antibody.

Figure 15 is a photographic representation of immunofluorescence analysis of L6/FLAG-Csl proliferating cultures with Hoechst nuclear stain (blue [photographs A and B]), anti-FLAG (red [photographs C, D, E and F]) or MF20 anti-muscle myosin (green [photographs G, H, I and J]) antibodies.

Figure 16 is a photographic representation of expression of myogenin mRNA in L6E9 (L6) cells and L6/IGF-I, L6/CSL (L6/Csl) and L6/IGF Csl cells at different times of differentiation. GM are myoblasts grown in growth medium. Times of differentiation represented in days. Note increased and prematurely activated expression of myogenin in L6/IGF Csl cells only.

Figure 17 is a photographic representation of calcineurin expression and subcellular localization are responsive to postmitotic IGF-I expression in myogenic cell cultures.

(A) Effects of MLC/IGF-I expression on CnA and CnB transcripts in differentiating L6E9 cultures. L6E9 or L6MLC/IGF-I myoblasts (13) were maintained in growth medium (GM) or shifted to differentiation medium (DM) for the number of days indicated. Total RNA from the cultures was harvested and analyzed by Northern blot first with a CnB probe to distinguish a characteristic 4.0kb CnB transcript (10) in all samples. Sequential hybridization with a CnA probe revealed two CnA transcripts, a minor ~4.5 kb species previously detected in skeletal muscle (10), and a predominant ~1.7 kb species characteristic of testis (10) which increased in response to IGF-I-induced hypertrophy.

(B) Effects of MLC/IGF-I expression on calcineurin protein levels and subcellular distribution in L6E9 myocytes. Panel a: Western blot analysis of 61kDa CnA protein in L6E9 (lane 1) or L6MLC/IGF-I (lane 2) differentiated myocytes harvested at d3 (DM), using an anti-CnA antibody. Panel b: Immunofluorescent analysis of calcineurin in the

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same cultures. Hoechst staining (top, blue) highlights the characteristic nuclear rings typical of hypertrophic myocytes in L6MLC/IGF-I differentiated cultures. This unusual phenotype may be related to defective sarcomeric reorganization in the hypertrophied fibers, previously visualized by the localization of skeletal actin in these cells (13). Anti-
5 CnA antibody (bottom, red) reveals the predominantly nuclear localization of calcineurin in the L6MLC/IGF-I hypertrophic myocytes.

Figure 18 is a photographic representation of perturbation of calcineurin action affects hypertrophy in myogenic cell cultures.

10

(A) Hypertrophic response to activated CnA expression in post-mitotic L6E9 myocytes. L6E9 myoblasts were stably transfected with a calcium-independent activated CnA mutant (35) under the control of a myogenin promoter to produce L6Myog/CnA cultures. Parental L6E9 and L6Myog/CnA cultures were differentiated for two days in DM. Phase
15 contrast photographs of representative fields from the two cultures are shown at 200x and 400x magnification. Note nuclear rings in the L6Myog/CnA myocytes, characteristic of IGF-I-induced hypertrophy.

(B) Suppression of myogenic differentiation by constitutive expression of a HA tagged,
20 dominant-negative CnA mutant (CnDN-HA) in transiently transfected L6MLC/IGF-I cultures (14). Transfectants were switched to DM for 2 days and the CnDN-HA mutant (red panels b and e) and muscle myosin (green panels c and f) were analyzed by immunofluorescence, with anti-HA and MF20 antibodies, respectively. Panels a-c show a field of HA-positive transfectants, panels d-f show a field of untransfected cells from the
25 same plate. Myocyte hypertrophy in the cultures, characterized by nuclear rings (Panel d) and muscle myosin expression (Panel f) was blocked in those cells transfected with the CnDN-HA plasmid (compare Panels b and c).

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(C) Cyclosporin blocks myogenic differentiation and hypertrophy. L6E9 and L6MLC/IGF-I cultures were differentiated in parallel in the presence of 5 μ M CsA, added either at d0 or d2 after the cells were switched to DM. CsA added at d0 blocked myogenic differentiation in both lines by d5, as visualized by immunofluorescent analysis of muscle myosin with an MF20 antibody. CsA added at d2 did not block myosin expression but reduced the extent of hypertrophy in the L6MLC/IGF-I myocytes (compare panels L6MLC/IGF-I untreated d5 DM and CsA d2-d5 DM). Similar experiments conducted with 3 μ M CsA yielded identical results.

10 **Figure 19** is a photographic representation of activation of GATA-2 expression in hypertrophic myocytes.

(A,B) Accumulation of GATA-2 transcripts in response to IGF-I and CnA action or inhibition. Panel A: Northern analysis of GATA-2 transcripts in L6E9 or L6MLC/IGF-I cultures maintained in GM, or differentiated in DM for up to 4 days. Panel B: Northern analysis of GATA-2 transcripts in L6E9 (lane 1), L6MLC/IGF-I (lane 2), L6MLC/IGF-I + 3 μ M CsA added at d0 (lane 3), and L6Myog/CnA cultures, after 2 days in DM (lane 4).

20 (C) Subcellular localization of GATA-2 protein in nuclei of L6MLC/IGF-I hypertrophic myocytes. L6E9 and L6MLC/IGF-I cultures were differentiated in DM for 3 days, then analyzed for GATA-2 protein by immunofluorescence (red - bottom panels). Top panels show nuclear Hoechst stain (blue). Of the three fields shown for L6MLC/IGF-I cultures, the two left-hand fields show GATA-2 localized to hypertrophic myocytes, characterized by nuclear rings, whereas the right-hand field is of a less densely seeded area from the same plate devoid of hypertrophic myocytes and GATA-2 protein.

(D) Subcellular localization of GATA-2 protein in nuclei of L6Myog/CnA hypertrophic myocytes. L6E9 and L6Myog/CnA cultures were differentiated in DM for 3 days, then analyzed for GATA-2 protein by immunofluorescence (red - bottom panels). Top panels

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show nuclear Hoeschst stain (blue). Note GATA-2 localization to hypertrophic myocytes, characterized by nuclear rings, exclusively in the L6Myog/CnA cultures.

Figure 20 is a graphical representation of the induction and calcineurin-dependence of IGF-1-mediated hypertrophy in C2C12 cells. **a**, Morphology of control (transfected with vector alone), IGF-1 transfected (IGF-1) and IGF-1 plus cyclosporin A (CsA, 1 μ M)-treated myotubes. CsA was added at the time of plating C2C12 myoblasts and differentiation induced 24 hours later. Myotubes, shown at five days post-differentiation, were fixed and stained with phalloidin to reveal *F*-actin fibres (bar=20 μ m). **b**, Mean area of control (open bars), IGF-1-(solid bars) and IGF-1 plus CsA-treated (hatched bars) five-day post-differentiation myotubes, and glycolytic enzyme activities (LDH, lactate dehydrogenase; ALT, alanine aminotransferase) and lactate levels for IGF-1 and IGF-1 plus CsA myotubes. The LDH, ALT and lactate values shown are the fold increases above the levels observed in control cells (LDH, $5.2 \pm 0.6 \times 10^{-3}$ BB units/mg protein; ALT, $0.34 \pm 0.01 \times 10^{-3}$ SF units/mg; lactate 6.4 ± 0.2 mg/dL). In all cases the myotube area, and also myotube width (data not shown), as well as glycolytic enzyme and lactate levels were significantly greater ($p < 0.001$) than the corresponding control and IGF-1 plus CsA values. **c**, [3 H]-thymidine incorporation and fractional protein synthesis rate in control (C), IGF-1 myoblasts and IGF-1 myotubes in the absence (open bars) or presence (solid bars) of CsA (1 μ M) (* $p < 0.01$ vs respective value in the presence of CsA).

Figure 21 is a graphical representation of calcineurin phosphatase (CnPP) activity in control (-O-), IGF-1- (-●-) or IGF-1 plus CsA- (-■-) treated myoblasts (B1, one day after plating) and myotubes (T1-T5, one, three or five days after changing to differentiation medium). *Inset*, CnA and CnB expression (immunoblot analysis; equal protein loading per lane) in control and IGF-1 treated myoblasts and myotubes.

Figure 22 is a graphical representation of the induction and calcineurin-dependence of C2C12 cell myotube hypertrophy resulting from insulin plus dexamethasone (I/D) treatment. **a**, Myotube width and lactate levels in five-day post-differentiation, normal

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(N), I/D- and I/D plus CsA-treated myotubes (* $p < 0.001$). **b**, Calcineurin phosphatase (CnPP) activity (100nM calmodulin) in N (-▲-) or I/D plus CsA(--■--) treated myoblasts or myotubes (left panel), or in control (-O-), IGF-1-transfected (-●-) or I/D-treated (-▲-) T1 myotubes at different concentrations of exogenously added calmodulin (right panel).

5

Figure 23 is a graphical representation of the effect of IGF-1-induced transformation of rat latissimus dorsi muscle (LDM) *in vivo*. **a**, Green fluorescence of IGF-1-transformed LDM (bar=10 μ m); **b**, lactate levels of control (C) and IGF-1-transformed (IGF-1) LDM. * $p < 0.001$. *Inset*, Northern blot analysis showing IGF-1 mRNA in control and pIGF-1/IRES-GFP20-treated LDM at 1,2 and 3 months post-injection (IGF-1 mRNA) from one control and two IGF-1-injected LDM samples are shown at each time point; equal amounts of RNA were loaded in all instances); **c**, Calcineurin phosphatase (CnPP) activity in control and IGF-1-transformed LDM (* $p < 0.001$); **d**, H&E stained control and IGF-1-injected LDM. Central nuclei were apparent in 3-10% of IGF-1 myofibres but were not detected in control myofibres (0%, $p < 0.001$) bar=10 μ m).

10

Figure 24 is a schematic representation of the murine Csl allele and the knockout allele. pgkNeopA is the neomycin resistance cassette. HPAP is a human placental alkaline phosphatase expression cassette.

20

Figure 25 is a photographic representation of Southern transfer showing screening of individual ES cell DNA for incorporation of the targeted Csl allele.

DETAILED DESCRIPTION OF THE INVENTION

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Accordingly, one aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence corresponding to a gene which is expressed in heart muscle from about 8 dpc in murine species or its equivalents in other mammalia species such as humans.

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More particularly, the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding, or a nucleotide sequence complementary to a nucleotide sequence encoding, an amino acid sequence substantially as set forth in SEQ ID NO:2 or a derivative or homolog or mimetic thereof or having at least about 45% or greater
5 similarity to at least 20 contiguous amino acids in SEQ ID NO:2 or a derivative or homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.

Even more particularly the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding, or a nucleotide sequence complementary to a nucleotide
10 sequence, encoding an amino acid sequence substantially as set forth in SEQ ID NO:4 or a derivative, homolog or mimetic thereof or having at least about 45% or greater similarity to at least 20 contiguous amino acids in SEQ ID NO:4 or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.

15 Yet even more particularly the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding, or a nucleotide sequence complementary to a nucleotide sequence encoding, an amino acid sequence substantially as set forth in SEQ ID NO:5 or a derivative, homolog or mimetic thereof or having at least about 45% or greater similarity to at least 20 contiguous amino acids in SEQ ID NO:5 or a derivative, homolog,
20 analog, chemical equivalent or mimetic of said nucleotide sequence.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids
25 that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. The percentage similarity may be greater than 50% such as at least 70% or at least 80% or at least 90% or at least 95% or
30 higher.

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To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences may be aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at
5 corresponding amino acid positions or nucleotide positions can then be compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e. % identity = # of identical positions/total
10 # of overlapping positions x 100). Preferably, the two sequences are the same length. The determination of percent identity or homology between two sequences can be accomplished using a mathematical algorithm. A suitable, mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc.*
15 *Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches can be performed with XBLAST program,
20 score = 50, wordlength = 3 to obtain amino acid sequences homologous to the protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See
25 <http://www.ncbi.nlm.nih.gov>. Another example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of
30 12, and a gap penalty of 4 can be used. The percent identity between two sequences can

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be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

Another aspect of the present invention contemplates a nucleic acid molecule comprising a
5 nucleotide sequence substantially as set forth in SEQ ID NO:1 or a derivative, homolog or
mimetic thereof capable of hybridising to SEQ ID NO:1 under low stringency conditions
at 42°C.

Still another aspect of the present invention contemplates a nucleic acid molecule
10 comprising a nucleotide sequence substantially as set forth in SEQ ID NO:3 or a
derivative, homolog or mimetic thereof capable of hybridising to SEQ ID NO:3 under
low stringency conditions at 42°C.

Reference herein to a low stringency at 42°C includes and encompasses from at least
15 about 1% v/v to at least about 15% v/v formamide and from at least about 1M to at least
about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for
washing conditions. Alternative stringency conditions may be applied where necessary,
such as medium stringency, which includes and encompasses from at least about 16% v/v
to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M
20 salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing
conditions, or high stringency, which includes and encompasses from at least about 31%
v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about
0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for
washing conditions. In general, washing is carried out at $T_m = 69.3 + 0.41 (G + C) \%$
25 $[19] = -12^\circ\text{C}$. However, the T_m of a duplex DNA decreases by 1°C with every increase
of 1% in the number of mismatched based pairs (7).

Preferably the present invention contemplates a nucleic acid molecule comprising a
nucleotide sequence substantially as set forth in SEQ ID NO:1 or a derivative, homolog or
30 mimetic thereof capable of hybridising to SEQ ID NO:1 under low stringency conditions

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at 42°C and which encodes an amino acid sequence corresponding to an amino acid sequence set forth in SEQ ID NO:2 or a sequence having at least about 45% similarity to at least 20 contiguous amino acids in SEQ ID NO:2.

- 5 Preferably the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in SEQ ID NO:3 or a derivative, homolog or mimetic thereof capable of hybridising to SEQ ID NO:3 under low stringency conditions at 42°C and which encodes an amino acid sequence corresponding to an amino acid sequence set forth in SEQ ID NO:4 or a sequence having at least about 45% similarity to
- 10 at least 20 contiguous amino acids in SEQ ID NO:4.

More particularly, the present invention contemplates a nucleic acid molecule comprising a sequence of nucleotides substantially as set forth in SEQ ID NO:1 or 3.

- 15 The nucleic acid molecule according to this aspect of the present invention corresponds herein to "Csl". This gene has been determined, in accordance with the present invention, to undergo expression in heart muscle from 8 dpc in normal mice but not in the hearts of mutants which lack the major determinant of cardiac development - Nkx2.5. It is thereby thought to form a novel member of the group of genes involved in muscle formation
- 20 and/or function and in particular heart formation and/or function. The product of the Csl gene is referred to herein as Csl. Murine Csl is defined by the amino acid sequence set forth in SEQ ID NO:2, human Csl is defined by the amino acid sequence set forth in SEQ ID NO:4 and Xenopus Csl is defined by the amino acid sequence set forth in SEQ ID NO:5. The cDNA nucleotide sequence for murine and human Csl are defined by the
- 25 nucleotide sequences set forth in SEQ ID NO:1 and SEQ ID NO:3, respectively. It should be understood that reference to the "characteristics" of Csl is a reference to the capacity of this molecule to perform a functional role in the regulation of muscle cell formation and/or functional activity.

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The nucleic acid molecule encoding *Csl* is preferably a sequence of deoxyribonucleic acids such as a cDNA sequence or a genomic sequence. A genomic sequence may also comprise exons and introns. A genomic sequence may also include a promoter region or other regulatory regions.

5

Another aspect of the present invention contemplates a genomic nucleic acid molecule or a derivative or homolog or mimetic thereof capable of hybridising to SEQ ID NO:1 or 3 or a derivative or homolog or mimetic thereof under low stringency conditions at 42°C.

10 Reference herein to *Csl* and *Csl* should be understood as a reference to all forms of *Csl* and *Csl*, respectively, including, for example, any peptide and cDNA isoforms which arise from alternative splicing of *Csl* mRNA or mutants or polymorphic variants of *Csl* or *Csl*. To the extent that it is not specified, reference herein to *Csl* and *Csl* includes reference to derivatives homologs, analogs, chemical equivalents and mimetics thereof.

15

The term "protein" should be understood to encompass peptides polypeptides and proteins. The protein may be glycosylated or unglycosylated and/or may contain a range of other molecules fused, linked, bound or otherwise associated to the protein such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins. Reference herein to a

20 "protein" includes a protein comprising a sequence of amino acids as well as a protein associated with other molecules such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins.

The protein and/or gene is preferably from a human, primate, livestock animal (eg. sheep, 25 pig, cow, horse, donkey) laboratory test animal (eg. mouse, rat, rabbit, guinea pig) companion animal (eg. dog, cat), captive wild animal (eg. fox, kangaroo, deer), aves (eg. chicken, geese, duck, emu, ostrich), reptile or fish.

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Derivatives include fragments, parts, portions, mutants, and mimetics from natural, synthetic or recombinant sources including fusion proteins. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. An example of substitutional amino acid variants are conservative amino acid substitutions. Conservative amino acid substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine. Additions to amino acid sequences including fusions with other peptides, polypeptides or proteins.

Homologs of the protein contemplated herein include, but are not limited to, proteins derived from different species.

Chemical and functional equivalents of *Csl* or Csl should be understood as molecules exhibiting any one or more of the functional activities of *Csl* or Csl and may be derived from any source such as being chemically synthesized or identified via screening processes such as natural product screening.

The derivatives of Csl include fragments having particular epitopes of parts of the entire Csl protein fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. For example, Csl or derivative thereof may be fused to a molecule to facilitate its entry into a cell.

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Analogues of Csl contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogues.

5

Derivatives of nucleic acid sequences may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules of the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in cosuppression and fusion of nucleic acid molecules. Derivatives of nucleic acid sequences also include degenerate variants.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH_4 ; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with NaBH_4 .

20

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

25 The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride

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or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

5

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

10

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carboethoxylation with diethylpyrocarbonate.

- 15 Examples of incorporating unnatural amino acids and derivatives during protein synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminoheptanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid contemplated
- 20 herein is shown in Table 1.

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TABLE 1

	Non-conventional amino acid	Code	Non-conventional amino acid	Code
5	α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
	α -amino- α -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
	aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasn
			L-N-methylaspartic acid	Nmasp
10	aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
	aminonorbornyl- carboxylate	Norb	L-N-methylglutamine	Nmgln
			L-N-methylglutamic acid	Nmglu
	cyclohexylalanine		Chexa L-N-methylhistidine	Nmhis
	cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
15	D-alanine	Dal	L-N-methylleucine	Nmleu
	D-arginine	Darg	L-N-methyllysine	Nmlys
	D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
	D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
	D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
20	D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
	D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
	D-isoleucine	Dile	L-N-methylproline	Nmpro
	D-leucine	Dleu	L-N-methylserine	Nmser
	D-lysine	Diys	L-N-methylthreonine	Nmthr
25	D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
	D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
	D-phenylalanine	Dphe	L-N-methylvaline	Nmval
	D-proline	Dpro	L-N-methylethylglycine	Nmetg
	D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
30	D-threonine	Dthr	L-norleucine	Nle

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	D-tryptophan	Dtrp	L-norvaline	Nva
	D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
	D-valine	Dval	α -methyl- γ -aminobutyrate	Mgab
	D- α -methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
5	D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
	D- α -methylasparagine	Dmasn	α -methyl- α -naphthylalanine	Manap
	D- α -methylaspartate	Dmasp	α -methylpenicillamine	Mpen
	D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
10	D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D- α -methylisoleucine	Dmile	N-amino- α -methylbutyrate	Nmaabu
	D- α -methylleucine	Dmleu	α -naphthylalanine	Anap
	D- α -methyllysine	Dmlys	N-benzylglycine	Nphe
	D- α -methylmethionine	Dmnet	N-(2-carbamylethyl)glycine	Ngln
15	D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D- α -methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
20	D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D- α -methyltyrosine	Dmtty	N-cyclodecylglycine	Ncdec
	D- α -methylvaline	Dmval	N-cylcododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
25	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
30	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser

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	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
5	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpn
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
10	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyl- α -naphthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
	L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
15	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L- α -methylalanine	Mala
	L- α -methylarginine	Marg	L- α -methylasparagine	Masn
	L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
	L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
20	L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
	L- α -methylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
	L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
	L- α -methylleucine	Mleu	L- α -methyllysine	Mlys
	L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
25	L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
	L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
	L- α -methylserine	Mser	L- α -methylthreonine	Mthr
	L- α -methyltryptophan	Mtrp	L- α -methyltyrosine	Mtyr
	L- α -methylvaline	Mval	L-N-methylhomophenylalanine	Nmhph
30	N-(N-(2,2-diphenylethyl)	Nnbhm	N-(N-(3,3-diphenylpropyl)	Nnbhe

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carbamylmethyl)glycine

carbamylmethyl)glycine

1-carboxy-1-(2,2-diphenyl-Nmbc

ethylamino)cyclopropane

5 Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with $n=1$ to $n=6$, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety.

10

The nucleic acid molecule of the present invention is preferably in isolated form or ligated to a vector, such as an expression vector. By "isolated" is meant a nucleic acid molecule having undergone at least one purification step and this is conveniently defined, for example, by a composition comprising at least about 10% subject nucleic acid

15 molecule, preferably at least about 20%, more preferably at least about 30%, still more preferably at least about 40-50%, even still more preferably at least about 60-70%, yet even still more preferably 80-90% or greater of subject nucleic acid molecule relative to other components as determined by molecular weight, encoding activity, nucleotide sequence, base composition or other convenient means. The nucleic acid molecule of the

20 present invention may also be considered, in a preferred embodiment, to be biologically pure.

In a particularly preferred embodiment the nucleotide sequence corresponding to *Csl* is a cDNA sequence comprising a sequence of nucleotides as set forth in SEQ ID NO:1 or is

25 a derivative, homolog, analog, chemical equivalent or mimetic thereof including a nucleotide sequence having similarity to SEQ ID NO:1.

In another particularly preferred embodiment, the nucleotide sequence corresponding to *Csl* is a cDNA sequence comprising a sequence of nucleotides as set forth in SEQ ID

30 NO:3 or is a derivative, homolog, analog, chemical equivalent or mimetic thereof

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including a nucleotide sequence having similarity to SEQ ID NO:3.

In yet another particularly preferred embodiment the nucleotide sequence corresponding to *Csl* is a genomic nucleotide sequence or a derivative, homolog or mimetic thereof
5 corresponding to a gene map as set forth in Figure 1.

In another particularly preferred embodiment the nucleotide sequence corresponding to *Csl* is a genomic nucleotide sequence or a derivative, homolog or mimetic thereof corresponding to a gene map as set forth in Figure 2.

10

In still yet another particularly preferred embodiment the nucleotide sequence corresponding to *Csl* is a genomic nucleotide sequence or a derivative, homolog or mimetic thereof having exon regions of which 5 comprise:

- 15 Exon 1 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:6;
Exon 2 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:7;
Exon 3 comprising a nucleotide sequence corresponding to the nucleotide
20 sequence substantially as set forth in SEQ ID NO:8;
Exon 4 comprising the nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:9; and
Exon 5 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:10,

25

or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.

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- Without limiting the present invention in any way, human genomic *Csl* comprises 5 exons which are present in human cosmid clones U228D4 and U112E8, corresponding to accession numbers U73509 and U73508, respectively. Exon 1 is defined by SEQ ID NO:6 which corresponds to the nucleotide sequence beginning at residue 19497 and
5 ending at residue 19327 of the human cosmid clone U228D4. Exon 2 is defined by SEQ ID NO:7 which corresponds to the nucleotide sequence beginning at residue 15687 and ending at residue 15631 of the human cosmid clone U228D4. Exon 3 is defined by SEQ ID NO:8 which corresponds to the nucleotide sequence beginning at residue 5220 and ending at residue 5134 of the human cosmid clone U228D4. Exon 4 is defined by SEQ
10 ID NO:9 which corresponds to the nucleotide sequence beginning at residue 35384 and ending at residue 35236 of the human cosmid clone U112E8. Exon 5 is defined by SEQ ID NO:10 which corresponds to the nucleotide sequence beginning at residue 4101 and ending at residue 3680 of the human cosmid clone U112E8.
- 15 A derivative of a nucleic acid molecule of the present invention also includes a nucleic acid molecule capable of hybridising to a nucleotide sequences set forth in one of SEQ ID NO:1 or 3 or to a nucleotide sequence corresponding to the gene maps set out in Figures 1 or 2 or to a nucleotide sequence comprising 5 exons as hereinbefore defined under low stringency conditions. Preferably, low stringency is at 42°C.
- 20 In another embodiment the present invention is directed to an isolated nucleic acid molecule encoding *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleic acid molecule, said nucleic acid molecule selected from the list consisting of:
- 25 (i) A nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence set forth in one or more of SEQ ID NO:2 or 4 or 5 or a derivative or homolog or mimetic thereof or having at least about 45% similarity to at least 20 contiguous amino acids in one or more of SEQ ID NO: 2 or 4 or 5 or a
30 derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide

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sequence.

(ii) A nucleic acid molecule comprising a nucleotide sequence substantially as set forth in one or more of SEQ ID NO:1 or 3 or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.

(iii) A nucleic acid molecule comprising a nucleotide sequence corresponding to a gene map as set forth in Figures 1 or 2 or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.

10

(iv) A nucleic acid molecule having exon regions of which 5 comprise:

Exon 1 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:6;

Exon 2 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:7;

Exon 3 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:8;

Exon 4 comprising the nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:9; and

Exon 5 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:10,

or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleic acid molecule.

(v) A nucleic acid molecule capable of hybridising under low stringency conditions at 42°C to the nucleotide sequence substantially as set forth in one or more of SEQ ID NO:1 or 3 or a derivative or homolog or mimetic thereof and encoding an amino acid sequence corresponding to an amino acid sequence as set forth in one

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or more of SEQ ID NO:2 or 4 or a derivative or homolog or mimetic thereof or a sequence having at least about 45% similarity to at least 20 contiguous amino acids in one or more of SEQ ID NO:2 or 4, respectively, or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleotide sequence.

5

- (vi) A nucleic acid molecule capable of hybridising to the nucleic acid molecule of paragraphs (i), (ii) or (v) under low stringency conditions at 42°C and encoding an amino acid sequence having at least about 45% similarity to at least 20 contiguous amino acids in one or more of SEQ ID NO:2 or 4.

10

- (vii) A nucleic acid molecule capable of hybridising to the nucleic acid molecule of paragraphs (iii) or (iv) under low stringency conditions at 42°C.

- (viii) A derivative, homolog, analog, chemical equivalent or mimetic of the nucleic acid molecule of paragraphs (i) or (ii) or (iii) or (iv) or (v) or (vi) or (vii).

15

The nucleic acid molecule may be ligated to an expression vector capable of expression in a prokaryotic cell (e.g. *E.coli*) or a eukaryotic cell (e.g. yeast cells, fungal cells, insect cells, mammalian cells or plant cells). The nucleic acid molecule may be ligated or fused or otherwise associated with a nucleic acid molecule encoding another entity such as, for example, a signal peptide, epitope tag, fluorescent tag, dimerisation motif, inhibitory motif, activation motif or regulatory motif.

The present invention extends to the expression product of the nucleic acid molecule hereinbefore defined.

The expression product is Csl having an amino acid sequence set forth in one or more of SEQ ID NO:2 or 4 or 5 or is a derivative, homolog, analog, chemical equivalent or mimetic thereof as defined above or is a derivative, homolog or mimetic having an amino acid sequence of at least about 45% similarity to at least 20 contiguous amino acids in the

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amino acid sequence as set forth in one or more of SEQ ID NO:2 or 4 or 5 or a derivative or homolog or mimetic thereof.

Another aspect of the present invention is directed to an isolated protein selected from the
5 list consisting of:

- (i) A protein having an amino acid sequence substantially as set forth in one or more
of SEQ ID NO:2 or 4 or 5 or derivative, homolog, analog, chemical equivalent or
mimetic thereof or a sequence having at least about 45% similarity to at least 20
10 contiguous amino acids in one or more of SEQ ID NO:2 or 4 or 5 or a derivative,
homolog or mimetic thereof.
- (ii) A protein encoded by a nucleotide sequence substantially as set forth in one or
more of SEQ ID NO:1 or 3 or a derivative, homolog or mimetic thereof or a
15 sequence encoding an amino acid sequence having at least about 45% similarity to
at least 20 contiguous amino acids in one or more of SEQ ID NO:2 or 4, or a
derivative, homolog or mimetic thereof.
- (iii) A protein encoded by a nucleotide sequence corresponding to a gene map
20 substantially as set forth in Figure 1 or 2 or a derivative, homolog or mimetic
thereof.
- (iv) A protein encoded by a genomic *Csl* nucleotide sequence having exon regions of
which 5 comprise:

25

Exon 1 comprising a nucleotide sequence corresponding to the nucleotide
sequence substantially as set forth in SEQ ID NO:6;

Exon 2 comprising a nucleotide sequence corresponding to the nucleotide
sequence substantially as set forth in SEQ ID NO:7;

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Exon 3 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:8;

Exon 4 comprising the nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:9; and

5 Exon 5 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:10,

or a derivative, homolog, analog, chemical equivalent or mimetic thereof.

10 (v) A protein encoded by a nucleic acid molecule capable of hybridising to the nucleotide sequence as set forth in one of SEQ ID NO:1 or 3 or a derivative or homolog or mimetic thereof under low stringency conditions at 42°C and which encodes an amino acid sequence substantially as set forth in SEQ ID NO:2 or 4, respectively, or a derivative or homolog or mimetic thereof or an amino acid
15 sequence having at least about 45% similarity to at least 20 contiguous amino acids in one or more SEQ ID NO:2 or 4.

(vi) A protein as defined in paragraphs (i) or (ii) or (iii) or (iv) or (v) in a homodimeric form.

20

(vii) A protein as defined in paragraphs (i) or (ii) or (iii) or (iv) or (v) in heterodimeric form.

The Csl of the present invention may be in multimeric form meaning that two or more
25 molecules are associated together. Where the same Csl molecules are associated together, the complex is a homomultimer. An example of a homomultimer is a homodimer. Where at least one Csl is associated with at least one non-Csl molecule, then the complex is a heteromultimer such as a heterodimer. A heteromultimer may include for example, another molecule capable of modulating muscle cell differentiation.

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Without limiting the present invention to any one theory or mode of action, Csl is a novel member (or mimic of a member) of the EF-Hand protein super family, which includes myosin lights chains, calmodulin, troponin C and calcineurin B subunit. These proteins act as calcium sensors or calcium dependent enzyme subunits for the activation of diverse signalling pathways mediating numerous cellular responses. Csl is expressed in differentiated cardiac and skeletal myocytes. During skeletal muscle development, the Csl gene is activated after cells have begun to differentiate, however Csl gene expression declines with age in this tissue. Activation of the Csl gene in skeletal muscle cell lines also occurs after the onset of differentiation. Accordingly, it is thought that in both heart and skeletal muscle, Csl functions in regulating aspects of muscle cell development or functional activities such as, but not limited to, differentiation and/or adaptive processes (for example, cellular hypertrophy and the maintenance of muscle homeostasis in striated [cardiac and skeletal] muscles).

Still without limiting the method or mode of action of the present invention, Csl acts on the insulin-like growth factor I (herein referred to as "IGF-I") dependent pathway of muscle cell hypertrophy. Differentiation of myoblasts over expressing Csl is delayed, and is accompanied by an increased susceptibility to cell death under certain culture conditions, indicating that precocious expression of Csl can be detrimental to normal muscle cell differentiation. In contrast, Csl over-expression enhances myocyte hypertrophy and induction of myogenin gene expression in a hypertrophic muscle cell model where the calcineurin-dependent signalling pathway is activated by IGF-I during differentiation.

Csl therefore collaborates with signalling pathways such as the calcineurin dependent signalling pathway, to modulate, for example, muscle cell development or functional activity such as gene expression, muscle differentiation and/or adaptive processes. For example, Csl collaborates with signalling pathways to participate in muscle differentiation and enhanced myocyte hypertrophy in response to extrinsic stimuli. This could occur through interactions with cytoskeletal elements and/or through calcineurin signalling

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intermediates, both of which can link physiological or hormonal stimuli with adaptive gene expression programs. Csl is thought to be a component of a signalling system that modulates the expression of genes in muscles since it can promote myogenic differentiation, and it is thought to be an important regulator of the myogenic program.

- 5 Csl is thought to function after withdrawal of myoblasts from the cell cycle, when it is thought to interact with elements of the cytoskeleton and to play a role in the differentiation of contractile cells.

- Csl is part of a monitoring system for maintenance of muscle homeostasis, a system
10 which may also function in development to coordinate muscle size and pattern. The myofilament is constantly monitoring itself, it grows larger if used heavily, and is lost if used less. Experimental data indicate that a feedback system, working at both the transcriptional and translational levels, ensures correct homeostasis. First, the level of myofilament protein in the heart remains constant for a particular set of physiological
15 parameters, despite natural or experimental variation in the level of transcription of individual myofilament genes. Second, enforced expression of skeletal α -actin in C2C12 myoblasts causes transcriptional activation of other thin filament genes. Third, mechanical stretch in cardiac and skeletal muscle, one of the key sensors of load, induces a complex series of signal transduction events which leads to gene expression and
20 muscular hypertrophy. Csl is thought to function in these events.

- The cloning and sequencing of this gene and its expression product now provides an additional gene for use in the prophylactic and therapeutic treatment of diseases such as those involving aberrant muscle cell development and functional activity such as aberrant
25 muscle cell differentiation, proliferation or hypertrophy. Accordingly, the present invention contemplates therapeutic and prophylactic uses of Csl amino acid and nucleic acid molecules, in addition to Csl agonistic and antagonistic agents, for the regulation of muscle cell development and other functional activity, such as for example, regulation of muscle cell proliferation, differentiation, gene expression and/or regulation of signalling
30 pathway activity.

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The present invention contemplates, therefore, a method for modulating expression of *Csl* in a mammal, said method comprising contacting the *Csl* gene with an effective amount of an agent for a time and under conditions sufficient to up-regulate or down-regulate or otherwise modulate expression of *Csl*. For example, *Csl* antisense sequences
5 such as oligonucleotides may be introduced into a cell to down-regulate the functional activity of *Csl*. Conversely, a nucleic acid molecule encoding *Csl* or a derivative thereof may be introduced to enhance the functional activity of *Csl* in any cell expressing the endogenous *Csl* gene.

10 The term "modulating" includes up-regulating or down-regulating. Accordingly, although the preferred method is to induce muscle cell development or other functional activity, the inhibition of muscle cell development or functional activity, for example by inducing the overexpression of *Csl* in myoblasts, may also be desired for example to reverse maladaptive hypertrophy.

15

An "effective amount" means an amount necessary to at least partly attain the desired response.

Reference to a "muscle cell" should be understood in its broadest sense to include
20 reference to any muscle cell irrespective of its stage of differentiation. For example, immature cells which are committed to differentiating along any muscle cell lineage (including cardiac muscle cell lineage, striated muscle cell lineage or a smooth muscle cell lineage) to a muscle cell (for example a myoblast) are encompassed within the scope of this definition. In another example, ageing, diseased or damaged muscle cells which
25 no longer function normally (for example cells which exhibit a reduced or a non-existent ability to contract) are also encompassed within the scope of this definition. Examples of muscle cells also include, but are not limited to, myocytes and myoblasts which are functioning or will function as cardiac muscle cells, striated muscle cells (also known as skeletal muscle cells) and smooth muscle cells.

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Reference to "muscle cell functional activity" should be understood as a reference to the functions which a muscle cell is capable of performing such as, but in no way limited to, one or more of proliferation, differentiation, maintenance of homeostasis and viability, migration, development of morphology and molecular mechanisms such as transcription,
5 translation and signal transduction. These activities may also be referred to as muscle cell "development".

"Differentiation" refers to the partial or complete maturation of a cell and may be evidenced, for example, by altered cell surface protein expression.

10

Another aspect of the present invention contemplates a method for modulating activity of *Csl* in a mammal, said method comprising administering to said mammal a modulating effective amount of an agent for a time and under conditions sufficient to increase or decrease *Csl* activity.

15

Modulation of said activity by the administration of an agent to a mammal can be achieved by one of several techniques, including but in no way limited to introducing into said mammal a proteinaceous or non-proteinaceous molecule which:

20

- (i) modulates expression of *Csl*;
- (ii) functions as an antagonist of *Csl*;
- (iii) functions as an agonist of *Csl*.

25

Said proteinaceous molecule may be derived from natural or recombinant sources including fusion proteins or following, for example, natural product screening. Said non-proteinaceous molecule may be, for example, a nucleic acid molecule or may be derived from natural sources, such as for example natural product screening or may be
30 chemically synthesised. The present invention contemplates chemical analogs of *Csl*

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capable of acting as agonists or antagonists of *Csl*. Chemical agonists may not necessarily be derived from *Csl* but may share certain conformational similarities. Alternatively, chemical agonists may be specifically designed to mimic certain physiochemical properties of *Csl*. Antagonists may be any compound capable of

5 blocking, inhibiting or otherwise preventing *Csl* from carrying out its normal biological functions. Antagonists include monoclonal antibodies specific for *Csl*, or parts of *Csl*, and antisense nucleic acids which prevent transcription or translation of *Csl* genes or mRNA in mammalian cells. Agonists and antagonists of *Csl* should be understood to include any molecule which synergises with *Csl* to either upregulate or downregulate,

10 respectively, its activity. For example, IGF-1 synergises with *Csl* to induce muscle cell hypertrophy.

Said proteinaceous or non-proteinaceous molecule may act either directly or indirectly to modulate the expression of *Csl* or the activity of *Csl*. Said molecule acts directly if it

15 associates with *Csl* or *Csl* to modulate the expression of *Csl* or *Csl*. Said molecule acts indirectly if it associates with a molecule other than *Csl* or *Csl* which other molecule either directly or indirectly modulates the expression of activity of *Csl* or *Csl*. Accordingly, the method of the present invention encompasses the regulation of *Csl* expression or *Csl* activity via the induction of a cascade of regulatory steps which lead to

20 the regulation of *Csl* or *Csl* expression or activity.

The *Csl*, *Csl* or agent used may also be linked to a targeting means such as a monoclonal antibody, which provides specific delivery of the *Csl*, *Csl* or agent to the target cells. In a preferred embodiment of the present invention, the *Csl*, *Csl* or agent used in the

25 method is linked to an antibody specific for said target cells to enable specific delivery to these cells.

Another aspect of the present invention contemplates a method of modulating muscle cell functional activity in a mammal said method comprising administering to said mammal

30 an effective amount of an agent for a time and under conditions sufficient to modulate the

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expression of a nucleotide sequence encoding *Csl* or sufficient to modulate the activity of *Csl*.

Yet another aspect of the present invention contemplates a method of modulating muscle
5 cell functional activity in a mammal said method comprising administering to said
mammal an effective amount of *Csl* or *Csl*.

Without limiting the present invention to any one theory or mode of action it is thought
that *Csl* binds preferentially to a subset of EF-Hand targets, most preferably, to the IQ
10 site of myosin heavy chains or to the calcineurin A catalytic subunit.

Accordingly, in a most preferred embodiment the present invention is directed to a
method of modulating muscle cell functional activity in a mammal said method
comprising administering to said mammal an effective amount of an agent for a time and
15 under conditions sufficient to modulate the expression of a nucleotide sequence encoding
Csl or sufficient to modulate the activity of *Csl* wherein said *Csl* expression product or
said *Csl* interacts, binds or otherwise associates with an EF-Hand target.

Most preferably said EF-hand target is the IQ site on a myosin heavy chain or the
20 calcineurin A catalytic subunit.

Yet another aspect of the present invention contemplates a method of modulating muscle
cell functional activity in a mammal said method comprising administering to said
mammal an effective amount of *Csl* or *Csl* wherein said *Csl* or the expression product of
25 said *Csl* interacts, binds or otherwise associates with an EF-Hand target.

Preferably said EF-hand target is an IQ site on a myosin heavy chain or the calcineurin A
catalytic subunit.

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Without limiting the operation of the present invention in any way, it is thought that the binding of Csl to the calcineurin A catalytic subunit modulates the calcineurin-dependent signalling pathway.

- 5 Accordingly, yet another aspect of the present invention is directed to a method of modulating cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of Csl or *Csl* for a time and under conditions sufficient to modulate the activity of one or more components of a calcineurin-dependent signalling pathway.

10

Preferably said cellular functional activity is muscle cell functional activity. Even more preferably said component is the calcineurin A catalytic subunit.

- In yet another aspect the present invention is directed to a method of modulating cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a nucleotide sequence encoding *Csl* or sufficient to modulate the activity of Csl wherein said *Csl* expression product or Csl modulates the activity of one or more of the components of a calcineurin dependent signalling pathway.

20

Preferably said cellular functional activity is muscle cell functional activity. Even more preferably said component is the calcineurin A catalytic subunit.

- Administration of the Csl, *Csl* or agent, in the form of a pharmaceutical composition, may be performed by any convenient means. Csl, *Csl* or agent of the pharmaceutical composition are contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the Csl, *Csl* or agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 μ g to about 10 mg of Csl or agent may be administered per kilogram of body weight per day. For example from

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about 0.1 μg -5 mg, 10 μg -5 mg or 100 μg -1 mg. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation. The Csl or agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intranasal, intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). With particular reference to use of Csl or agent, these peptides may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with calcium, magnesium, zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

A further aspect of the present invention relates to the use of the invention in relation to mammalian disease conditions. The present invention is particularly useful, but in no way limited to, use in attenuating muscle frailty, treating muscular and myotonic dystrophies the prevention of cardiomyopathy, treating maladaptive hypertrophy, modulating myogenic adaptation in ageing muscle, modulating cytoskeletal signalling in myopathic muscle, modulating calcineurin signalling in heart and skeletal muscle or enhancing compensatory hypertrophy in heart failure due to infarction and/or dilated cardiomyopathy.

For example, the decline in function and restriction of adaptability of skeletal muscle is a hallmark of ageing in humans, leading to severe deficit in performance and ultimately to frailty. Maintenance of muscle integrity via modulation of Csl expression either alone or in concert with hormonal stimulation provides an approach for the prevention of

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muscle atrophy in ageing patients.

Muscular dystrophies are characterised by progressive weakness in specific muscle groups, leading to deterioration of muscle ultrastructures. Many of these pathologies arise from genetic defects that effect muscle integrity. Modulation of Csl activity provides an approach for counteracting the deterioration of muscle structure in some dystrophic patients. The inability to regenerate muscle tissue is another serious complication in muscular dystrophy. By utilising Csl as the therapeutic molecule the integrity of newly formed muscle cells can be preserved thereby counteracting muscle degeneration. Csl therapy is particularly attractive in gene therapy programs due to its small size and suitability for insertion into gene therapy vectors.

Yet another example of the use of the present invention relates to cardiomyopathy. Hypertrophy, whether familial or generated by hypertension or other idiopathic causes, is a normal adaptation to increased work load in the heart during which there is new myofilament protein synthesis and deposition of new myofilament proteins into the contractile apparatus. However, if the adaptive response is excessive, the heart becomes unable to cope with the changes, and both myofibrillar and myocyte disarray result, with a high risk of sudden death for the individual. Csl functions in organising myofilaments for the purpose of protecting or elevating the pathology associated with cardiac hypertrophy.

In another example, calcineurin signalling in heart and skeletal muscle could be modulated through direct interaction with the CnA catalytic subunit. The composition of active calcineurin, as analyzed historically in T lymphocytes and neurons, typically comprises a heterotrimer between the CnA catalytic and CnB regulatory subunits with Ca^{2+} -activated calmodulin. The importance of the calcineurin pathway in tissues other than the immune and nervous systems has only recently begun to be recognized. Alternate isoforms of calcineurin subunits could preferentially incorporate Csl as an alternate regulatory partner. Csl could shift the substrate specificity of calcineurin

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enzymatic activity in striated muscle under various conditions, in which case Csl would play a key role in the beneficial or detrimental effects of calcineurin signalling in these tissues.

- 5 The intense focus during the last decade on compounds which act as immunosuppressants by blocking calcineurin signalling in T lymphocytes underscores the potential clinical importance of a novel target for drugs to mediate this critical pathway. The currently available pharmacological inhibitors of calcineurin phosphatase, cyclosporin A and FK506, are directed against the regulatory CnB subunit in the calcineurin heterotrimer
- 10 responsible for T lymphocyte activation. Although these drugs are in wide use, their adverse side effects, such as renal impairment, hypertension and metastatic cancer, are serious.

- A novel application of these drugs has been recently proposed in the modulation of
- 15 human hypertrophic cardiomyopathies, since cardiac hypertrophy can be experimentally induced by expression in transgenic mouse myocardium of an activated form of calcineurin. Furthermore, hypertrophy associated with this and some other transgenic mouse model can be temporarily blocked by administration of cyclosporin A or FK506. It seems clear, however, from additional animal studies that these compounds will not be
- 20 useful in all hypertrophy situations, particularly that associated with pressure overload induced by hypertension. Csl, due to its cardiac and skeletal muscle specificity of expression, comprises a more directed target for pharmacological intervention in calcineurin signalling. In the heart, this could be either to abrogate undesirable hypertrophy in the case of hypertrophic cardiomyopathies, or to enhance compensatory
- 25 hypertrophy in the case of heart failure due to infarction and/or dilated cardiomyopathy. In skeletal muscle, it could be to enhance myogenic adaption and regeneration during ageing or in myopathies as detailed above.

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Accordingly, utilising the methods of the present invention, mammals may be treated for disorders including, but not limited to, diabetic cardiomyopathy, cardiac hypertrophy (acquired or familial), heart failure, dilated cardiomyopathy, myocarditis, loss of myofibers and loss of regenerative capacity in ageing and skeletal myopathies such as
5 Duchenne muscular dystrophy and Becker's myotonic dystrophy or myofiber atrophy.

As a potential regulatory molecule mapping to the X chromosome, *Csl* represents a candidate for X-linked genetic disorders affecting heart and skeletal muscle. Screens for polymorphisms in the *Csl* locus within populations carrying unmapped X-linked diseases
10 would be highly informative.

As a gene therapy tool, the relatively small size of the *Csl* protein makes it highly tractable as a gene therapeutic agent. In the heart, the potential reversal of cardiomyopathic syndromes by *Csl* gene introduction into postmitotic cells, inducing
15 compensatory hypertrophy or realignment of cardiomyocytes, represents an important advance in treatment of these common and often lethal pathologies. If warranted by the biology of *Csl* the gene could be readily and permanently delivered with AAV-based viral technologies, to a variety of postmitotic heart and skeletal muscle targets. In vivo expression of *Csl* may also be utilized, for example to screen for or otherwise test
20 proteinaceous or non-proteinaceous molecules for their capacity to function as agonists, antagonists or to otherwise modulate *Csl* expression or the *Csl* expression product's functional activity.

Accordingly, another aspect of the present invention relates to a method of treating a
25 mammal said method comprising administering to said mammal an effective amount of an agent capable of modulating the expression of *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate muscle cell functional activity.

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In another aspect the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of an agent capable of modulating the activity of *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate
5 muscle cell functional activity.

In another aspect the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and
10 under conditions sufficient to modulate muscle cell functional activity.

Yet another aspect the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and
15 under conditions sufficient to modulate muscle cell functional activity.

In yet another aspect the present invention relates to the use of an agent capable of modulating the expression of *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof in the manufacture of a medicament for the modulation of muscle cell
20 functional activity.

Another aspect of the present invention relates to the use of an agent capable of modulating the expression of *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof in the manufacture of a medicament for the modulation of muscle cell
25 functional activity.

A further aspect of the present invention relates to the use of *Csl* or *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof in the manufacture of a medicament for the modulation of muscle cell functional activity.

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Still yet another aspect of the present invention relates to agents for use in modulating *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof wherein modulating said *Csl* modulates muscle cell functional activity.

- 5 A further aspect of the present invention relates to agents for use in modulating *Csl* expression or a derivative, homolog, analog, chemical equivalent or mimetic thereof wherein modulating expression of said *Csl* modulates muscle cell functional activity.

- Another aspect of the present invention relates to *Csl* or *Csl* or a derivative, homolog,
10 analog, chemical equivalent or mimetic thereof for use in modulating muscle cell functional activity.

Preferably said muscle cell functional activity is heart or skeletal cell differentiation and even more preferably heart cell functional activity.

15

In a related aspect of the present invention, the mammal undergoing treatment may be human or an animal in need of therapeutic or prophylactic treatment.

- In accordance with these methods, *Csl*, *Csl* or agents capable of modulating *Csl*
20 expression or *Csl* activity or derivatives, homologs, analogs, chemical equivalents or mimetics thereof may be coadministered with one or more other compounds or molecules. For example, *Csl* may be coadministered with IGF-I. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or
25 different routes. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

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In yet another further aspect the present invention contemplates a pharmaceutical composition comprising *Csl*, *Csl* or an agent capable of modulating *Csl* expression or *Csl* activity or derivative, homolog, analog, chemical equivalent or mimetic thereof together with one or more pharmaceutically acceptable carriers and/or diluents. *Csl*, *Csl* or said agent are referred to as the active ingredients.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimersal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable

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solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

- 5 When *Csl*, *Csl* and *Csl* modulators are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the
- 10 form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful
- 15 compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μ g and 2000 mg of active compound.

- The tablets, troches, pills, capsules and the like may also contain the following: A
- 20 binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may
- 25 contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or
- 30 orange flavour. Of course, any material used in preparing any dosage unit form should

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be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

- 5 Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is
10 contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers
15 to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the
20 particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired.

The principal active ingredient is compounded for convenient and effective administration
25 in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5 μ g to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.5 μ g to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active
30 ingredients, the dosages are determined by reference to the usual dose and manner of

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administration of the said ingredients.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule
5 capable of modulating *Csl* expression or *Csl* activity. The vector may, for example, be a viral vector. The vector may comprise, for example, a muscle specific promoter, the *Csl* coding region and a polyadenylation sequence.

Still another aspect of the present invention is directed to antibodies to *Csl* or *Csl*
10 including catalytic antibodies. Such antibodies may be monoclonal or polyclonal and may be selected from naturally occurring antibodies to *Csl* or may be specifically raised to *Csl*. In the case of the latter, *Csl* may first need to be associated with a carrier molecule. The antibodies and/or recombinant *Csl* of the present invention are particularly useful as therapeutic or diagnostic agents. Alternatively, fragments of
15 antibodies may be used such as Fab fragments. Furthermore, the present invention extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies. The antibodies of this aspect of the present invention are particularly useful for immunotherapy and may also be used as a diagnostic tool or for monitoring the program
20 of a therapeutic regime.

Csl can also be used to screen for naturally occurring antibodies to *Csl* or *Csl*.

Specific antibodies can be used to screen for *Csl* proteins. The latter would be
25 important, for example, as a means for screening for levels of *Csl* in a cell extract or other biological fluid or purifying *Csl* made by recombinant means from culture supernatant fluid. Techniques for the assays contemplated herein are known in the art and include, for example, sandwich assays, ELISA and flow cytometry.

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It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal or fragments of antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An
5 antibody as contemplated herein includes any antibody specific to any region of Csl.

Both polyclonal and monoclonal antibodies are obtainable by immunization with the protein or peptide derivatives and either type is utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are
10 less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of Csl, or antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of
15 the product.

The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing
20 an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example Douillard and Hoffman, Basic Facts about Hybridomas, in *Compendium of Immunology* Vol II, ed. by Schwartz, 1981; Kohler and Milstein, *Nature* 256: 495-499, 1975; *European Journal of Immunology* 6: 511-519, 1976).

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Preferably, the antibody of the present invention specifically binds the Csl protein or Csl or derivative, homolog, analog, chemical equivalent or mimetic thereof. By "specifically binds" is meant high avidity and/or high affinity binding of an antibody to a specific antigen. Antibody binding to its epitope on this specific antigen is stronger than binding
30 of the same antibody to any other epitope, particularly those that may be present in

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molecules in association with, or in the same sample, as the specific antigen of interest. Antibodies that bind specifically to a polypeptide of interest may be capable of binding other polypeptides at a weak, yet detectable, level (e.g., 10% or less of the binding shown to the polypeptide of interest). Such weak binding, or background binding, is
5 readily discernible from the specific antibody binding to the polypeptide of interest, e.g. by use of appropriate controls.

Another aspect of the present invention contemplates a method for detecting Csl or *Csl* in a biological sample from a subject.

10

In one preferred embodiment the present invention contemplates a method for detecting Csl in a biological sample from a subject said method comprising contacting said biological sample with an antibody specific for Csl or its derivatives or homologs for a time and under conditions sufficient for an antibody-Csl complex to form, and then
15 detecting said complex.

The presence of Csl may be determined in a number of ways such as by Western blotting, ELISA or flow cytometry procedures. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the
20 traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique
25 exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule
30 capable of producing a detectable signal is then added and incubated, allowing time

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- sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by
- 5 comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention the sample is one which might contain Csl
- 10 including cell extract, tissue biopsy or possibly serum, saliva, mucosal secretions, lymph, tissue fluid and respiratory fluid. The sample is, therefore, generally a biological sample comprising a tissue sample or biological fluid but also extends to fermentation fluid and supernatant fluid such as from a cell culture.
- 15 In the typical forward sandwich assay, a first antibody having specificity for the Csl, Csl or antigenic parts thereof, is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other
- 20 surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes) and under suitable conditions
- 25 (e.g. 25°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the hapten. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the hapten.

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- An alternative method involves immobilizing the target molecules in the biological sample and then exposing the immobilized target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.
- 10 By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and
- 15 chemiluminescent molecules.
- In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily
- 20 available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to
- 25 employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked
- 30 to the second antibody, giving a qualitative visual signal, which may be further

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quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

5

Alternately, fluorescent compounds, such as fluorecein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by
10 emission of the light at a characteristic color visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques
15 are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

The present invention also contemplates genetic assays such as involving PCR analysis
20 (for example, on DNA isolated from human blood or tissue) to detect *Csl* or its derivatives, homologs, polymorphisms or mutants.

Methods of detecting *Csl* or *Csl* may be utilized, for example, to quantitatively or qualitatively detect *Csl* or *Csl* levels. These methods may also be utilized to screen for
25 mutations or polymorphisms in *Csl* or *Csl* which mutations may result in, for example, aberrant functional activity of *Csl* or *Csl*. These methods may be utilized for the purpose of diagnosing or monitoring the progress of disease conditions characterized by aberrant *Csl* or *Csl* molecules, levels and/or functional activity. For example, the upregulation of *Csl* or *Csl* may be screened for in a mammal experiencing or predisposed to heart failure.
30 In another example, mutations in *Csl* or *Csl* molecules which result in over-activity of

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Csl or dominant-negative activity (wherein some aspects of Csl functional activity are altered but not others) may be screened for in individuals exhibiting symptoms of myopathy.

- 5 Yet another aspect of the present invention is directed to a method of diagnosing or monitoring a disease condition in a mammal, which disease condition is characterized by aberrant muscle cell functional activity, said method comprising screening for Csl and/or Csl in a biological sample isolated from said mammal.
- 10 Further features of the present invention are more fully described in the following non-limiting Figures or examples.

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SUMMARY OF SEQ ID Nos.

Sequence	SEQ ID NO.
cDNA nucleotide sequence of murine Csl	1
5 amino acid sequence of murine Csl	2
cDNA nucleotide sequence of human Csl	3
amino acid sequence of human Csl	4
amino acid sequence of Xenopus Csl	5
human genomic Csl sequence - Exon 1	6
10 human genomic Csl sequence - Exon 2	7
human genomic Csl sequence - Exon 3	8
human genomic Csl sequence - Exon 4	9
human genomic Csl sequence - Exon 5	10

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EXAMPLE 1

CLONING AND SEQUENCE ANALYSIS

In order to isolate novel genes that may be regulated by Nkx2.5 in the heart a modified
5 form of representational difference analysis (RDA) 'PCR-select' (Clontech Ltd) was
used. Mice heterozygous for the mutant allele Nkx2.5-/+ were intercrossed and
pregnant mothers were killed at 8.5 days post-coitum (dpc). Heart tissue was isolated
from each fetus and RNA was extracted. The remaining body tissue was used to isolate
DNA for PCT genotyping. Differential hybridisations were performed according to the
10 manufacturers instructions using cDNA synthesised from Nkx2.5-/- heart RNA as the
driver and heart RNA wild-type for Nkx2.5 as the tester. The resulting PCR bands were
isolated, cloned, sequenced and hybridised against the original cDNA isolates to
determine differential expression. It was established that one of these products (Cs1) is
found in normal fetal heart cDNA but is either very low or absent from Nkx2.5-/- heart
15 cDNA. The partial cDNA clone was compared against sequence databases and a series
of expressed sequence tags (ESTs) were found to match at highly significant levels.
These are listed in Table 2. These ESTs were derived from random sequencing projects
of fetal mouse heart and skeletal muscle and human heart and skeletal muscle cDNA
libraries. By compiling contigs of ESTs it was possible to predict a full length spliced
20 product for mouse and human genes. Primers were designed to the termini of the
predicted mouse cDNA and a product was amplified by PCR of the correct size from
fetal heart cDNA. This was cloned, sequenced and confirmed as identical to the EST
contig. The open reading frame (ORF) of the mouse sequence encodes a short protein of
86 amino acids which has no significant homology to any known protein in the current
25 databases. The small ORF was compared with the predicted human cDNA. The human
cDNA has only one ORF of reasonable length that could encode a similar protein to the
mouse. The human sequence encodes an 87 amino acid polypeptide that is 86% identical
to the mouse (Figure 3). The human gene contains 5 exons covering 50 kb and the splice
sites, splice orientation and exon sequence matches have been predicted from the EST
30 cDNA configuration.

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The mouse cDNA clone was used to isolate several cDNA clones from a *Xenopus* cDNA library.

EXAMPLE 2

CHISEL GENE EXPRESSION

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Among adult tissues, heart and skeletal muscles are the predominant sites of expression (Fig. 4), with both slow and fast twitch muscles showing robust levels of transcript (Fig. 5). Expression of Chisel does not appear to be modified in renal artery-banded rat hearts which have undergone cardiac hypertrophy (Fig. 6). During skeletal muscle development, the *Csl* gene is activated after cells have begun to differentiate, however *Csl* gene expression declines with age in this tissue. Activation of the *Csl* gene in skeletal muscle cell lines also occurs after the onset of differentiation. Thus, it is believed that in both heart and skeletal muscle, *Csl* functions in regulation aspects of differentiation or adaptive processes that maintain muscle homeostasis.

The distribution of *Csl* mRNA during early mouse development was analysed in detail using whole mount *in-situ* hybridisation with an RNA probe synthesised from the full length cDNA. Message was detected in the cardiac crescent at 8.0 dpc which persists into the linear heart tube stage. During heart looping, the expression is seen exclusively in the myocardial layer of the outer curvature of the presumptive left and right ventricles. Expression can be seen later in discrete regions of the developing atria but remained off in atrioventricular canal. *Csl* is a novel marker for these regions since this expression pattern has not been previously reported. It is believed that *Csl* expression may define the subpopulation of cells that form the definitive contractile tissue of the ventricles and atria.

In developing limb buds, *Csl* mRNA can be detected as early as 12.5 dpc by RNase protection analysis.

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An analysis has been performed of Csl expression in myogenic cell lines, which can be propagated in high serum as undifferentiated myoblasts, or induced to withdraw from the cell cycle and differentiate by removal of serum. In the robust and commonly used C2C12 line, thought to represent adult satellite cells, Csl was strongly expressed only
5 after myogenic differentiation, further underscoring its role in post-mitotic muscle function. Thus, Csl appears to function in the differentiation phase of muscle development. Csl was not expressed in a compromised myogenic cell line, L6E9, derived from neonatal limb muscle. L6E9 cells differentiate very poorly and only fuse into myotubes after long periods in culture. However, in the presence of IGF1, which is
10 normally expressed in the microenvironment of developing muscles, differentiation and fusion is dramatically improved. In the presence of IGF1, Csl is activated, although very weakly compared to levels in C2C12 cells.

Csl RNA was also present at very low levels in quiescent 10T1/2 fibroblasts, but absent
15 from all proliferating cell types analyzed to date.

Csl expression is also analysed by *in-situ* hybridisation to sectioned material. Firstly, sections of later stage mouse fetuses are examined for the changing pattern of Csl distribution in the developing heart. As in earlier hearts, expression was restricted to the
20 working myocardium at the atria and ventricles.

EXAMPLE 3

TARGETED DISRUPTION OF THE CSL ALLELE

25 The cosmid clones (U228D4 and U112E8) containing the genomic sequences of human CSL were derived from the long arm of the human X chromosome. X-unique genes are known to be highly conserved on the X chromosomes of all mammals due to the constraints of dosage compensation (8). Mouse Csl has been mapped to the mouse X by inter-specific back-cross analysis (Stanley *et al.* (1998) *Genomics* 49:337-338; Cpoeland
30 and Jenkins (1991) *Trends Genet.* 7:113-118). Csl maps near a gene called Gja6 that

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maps on human chromosome Xq 21.3 - q 22. Human Csl maps on Xq 22.1- q 22.2.

EXAMPLE 4

ANALYSIS OF CSL PROTEIN LOCALISATION IN FIBROBLAST AND MUSCLE CELLS

The Csl protein appears to be tethered to a component of the cytoskeleton, with the pattern in fibroblasts resembling that of the microfilament or intermediate filament networks. To produce a form of chisel protein detectable in cells the cDNA was cloned
10 as a fusion with the "flag" epitope in pEFBOS. This construct was transiently transfected into cos cells which were fixed, permeabilised and stained using anti-flag antibodies. In these experiments, staining was found in both the nuclei and the cytoplasm.

Stable 10T1/2 transfectants were made using the same construct and 24 independent
15 clones were isolated. In these cell lines, Csl appeared to be present in high density in and around the nucleus and in a gradually diminishing concentration out to the periphery of the cell. In the distal regions of the cytoplasm where the staining was weakest, fluorescence appeared to be filamentous and was often stronger near sites of cytoplasmic projections. In 10T1/2, C2C12 and 3T3 cells, Csl protein is localized to the nucleus
20 and, depending on the cell type, in a perinuclear region of the cytoplasm. Under appropriate fixation conditions, the cytoplasmic expression appears lacy. In transfected muscle cells, FLAG-Csl also appears to localize to the nucleus and perinuclear region, suggesting that this may be its normal location within the cell, although the pattern in muscle cells may be influenced by the distribution of myosin (see Example 5).

25

The stable 10T1/2 cell lines are stained with anti-flag antibodies after exposure of the cells to drugs that disrupt specific elements of the cytoskeleton: cytocholasin B which disrupts actin microfilaments and colcemid which disrupts microtubules. These cells are easily induced to fuse and form myotubes in culture whereupon the deposition of flag-Csl
30 is visualised.

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Using drug treatments as above, cytocholasin B for actin, colcemid for microtubules, it has been shown that the localization is dependent upon an intact actin cytoskeleton, but not upon intact microtubules. The binding of expressed Csl protein to the actin structures appears weak, since the pattern is disrupted if Triton is included during fixation.

5

EXAMPLE 5

COLOCALIZATION OF CSL AND MYOSIN HEAVY CHAINS

In differentiated L6E9/IGF-I lines transfected with FLAG- Csl, the myofilaments of some cells become unstable and myosin heavy chains localize unevenly in concentrated deposits within the cytoplasm. In these cases, Csl co-localizes with those deposits, suggesting that Csl binds to myosin heavy chains. This is consistent with the structural prediction (see Example 7).

10 To extend this observation further, Csl and/or a rat cardiac myosin heavy chain cDNAs have been expressed in COS cells utilising the Flag-Csl vector. When expressed in fibroblasts, myosin heavy chains self-assemble into thick filament-like rods that can readily be detected by immuno-histochemistry. When co-expressed, however, it has been observed that Csl co-localizes with the myosin heavy chain rods. Collectively, the data indicate that Csl can associate with myosin heavy chains in cultured cells. Further evidence for an interaction in vivo comes from the observation that in Csl-expressing L6E9 and C2C12 cells, the level of myosin heavy chains, as well as calcineurin A subunit, another potential target, are increased a few fold over levels in control cells (Figs. 7, 8).

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EXAMPLE 6
NUCLEAR LOCALIZATION OF TAGGED CSL EXPRESSED
IN POSTMITOTIC MYOCYTES

5 Since Csl is normally expressed exclusively in differentiated skeletal muscle, a differentiation-restricted Csl expression vector was constructed in order to mimic more accurately the activation pattern of the endogenous gene. In this vector (MLC/myc-Csl), the Csl protein is tagged with a myc epitope, and driven by the MLC1 promoter and downstream enhancer from the MLC1/3 locus. Immunofluorescent analysis of
10 transfected myc-Csl, expressed exclusively in differentiated L6E9 cultures, revealed a predominantly nuclear localization, with some association with unevenly distributed cytoplasmic myosin filaments (Fig. 9,10).

EXAMPLE 7
STRUCTURAL FEATURES OF CSL PROTEIN

15

The amino acid sequence of chisel has no significant homology to any known proteins in the database. However, its small size and the absence of cysteine residues makes it a good candidate for analysis by nuclear magnetic resonance (NMR) to solve the 3
20 dimensional structure. The GST-Csl fusion construct is used in a large scale protein production for NMR spectroscopy.

The primary structure of Csl was analyzed by homology screens with nucleic acid and protein sequence data bases. Csl bears no sequence homology with any other known
25 proteins in the public databases GenBank and EMBL. However, a structural prediction algorithm, Threader, detected structural homology between Csl and scallop regulatory myosin light chain. Myosin light chains are bi-lobal structures composed of four EF-Hand motifs (two per lobe), which are helix-turn-helix folds used in a variety of different proteins for binding Ca^{2+} or other divalent cations. Csl is only long enough to fold into
30 2 EF-Hands, therefore corresponding to only one lobe of the bi-lobal myosin light chain.

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The presence and position of the four helices of the 2 EF-Hands in Csl has been predicted by one of the BioMolecular Engineering Research Center (BMERC) structural algorithms (Protein Sequence Analysis System) (Fig. 11). Threader predicted that Csl resembles specifically the N-terminal lobe of the scallop regulatory myosin light chain, which binds a site in scallop myosin heavy chain related to, but distinct from that of the C-terminal lobe. The N- and C-terminal lobes bind to different regions of the so-called "incomplete" IQ sites on myosin heavy chains (Fig. 12). Essential light chains bind by a different modality to related but distinct "complete" IQ sites. The key residues in the interacts of EH-Hand proteins with their binding sites have been predicted from various structural determinations. By aligning potential target sites, it has been found that the key residues in the apparent Csl binding site on scallop myosin heavy chain are well conserved among other myosin heavy chains, but less so among calmodulin binding sites (Non-IQ motifs; Fig. 12).

Other proteins that contain EF-Hands include calmodulin (CaM), the major Ca^{2+} sensor in cells and a subunit of many Ca^{2+} -dependent proteins, troponin C, and the B subunit of the serine/threonine phosphatase calcineurin (CnB). It is noteworthy that CaM binds to its targets in a fashion different from myosin light chains. Structural modelling of Csl based on the structures of calmodulin and calcineurin B is performed. It was determined that in *in vitro* affinity assays, Csl has a negligible affinity for CaM binding motifs in such target proteins as CaMKinaseII and MLCKinase, but displays selective affinity for the calcineurin A (CnA) catalytic subunit.

It is thought that Csl serves as a specific regulatory subunit for calcineurin in cardiac and skeletal muscle. The B subunit structure has been determined and mutagenesis of CnA confirms the position of B subunit binding (Fig. 12). Notably, the proposed Csl binding site resembles more closely the CnB binding site than the CaM binding site on the CnA subunit.

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The relative importance of domains within the chisel protein can be indicated by interspecific comparisons and the alignment of mouse and human with *Xenopus* sequences. Comparisons between mouse, human and frog Csl suggest that the N-terminal region is more conserved than C-terminal and central regions. A series of mutations across the Csl protein are made and their characteristics in cell culture assays assessed. Their localization within the cell (cytoskeletal tethering; nuclear/cytoplasmic localisation; co-localization with myosin), their ability to enhance differentiation and fusion in C2C12 and L6MLC/IGF-I cells in low serum, and their ability to induce precocious differentiation in L6MLC/IGF-I cell in high serum are assessed. Mutant proteins are also assessed for their ability to bind to individual cellular or cytoskeletal components, known and unknown, *in vitro*. Site-directed mutagenesis is performed using the QuickChange system (Stratagene Ltd). Mutations are introduced into the FLAG-Csl and GST-Csl expression vectors and transfected into cell lines, or used for *in vitro* binding assays, respectively.

15

EXAMPLE 8

GENERATION OF CHISEL ANTIBODIES

Csl was expressed in bacteria as a glutathione-S-transferase (GST) fusion protein with GST fused at the N-terminus. The protein appears stable and Csl can be released from immobilized GST-Csl by the protease thrombin (Fig. 13). This product has the expected size as judged by mass spectroscopy. Thrombin and bacterial contaminants are normally removed by passage of protein through a 38kD pore-size spin column. Polyclonal antisera has been raised in rabbits against cleaved bacterial Csl, and this antisera detects transfected Csl and FLAG-csl in COS and C2C12 cell extracts (Fig. 14). Expressed Csl runs in SDS PAGE larger than bacterial Csl, indicative of post-translational modification or dimerisation.

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Determination of *Csl* subcellular localization is possible using the polyclonal antibody and monoclonal anti-*Csl* antibodies which are raised to bacterial *Csl* and synthetic *Csl* peptides.

- 5 The 10T1/2 and C2C12 Flag-*Csl* stable cell lines are also used in coimmunoprecipitation assays to determine if *Csl* binds to known proteins. Sepharose bound flag antibody are used to precipitate the Flag-*Csl* fusion protein from cell lysates and the resulting products electrophoresed. The coimmunoprecipitates are visualised initially by (35)S methionine labelling. A wide range of antibodies directed against potential binding partners are
10 tested on western blots of these coimmunoprecipitates.

EXAMPLE 9

IN-VITRO ASSAYS OF *Csl* FUNCTION

- 15 The *Csl* targeted ES cell line that is used to create null mutant mice is also differentiated in culture to produce embryoid bodies. Under these conditions it is possible to induce considerable levels of myogenic differentiation. By exposure to Cre through transfection of a Cre construct the single targeted *Csl* locus is inactivated and the effect of *Csl* loss on myogenic differentiation observed by testing for levels of specific markers and the effect
20 on cell phenotype. The presence of Cre-excised cells is assayed by alkaline phosphatase production.

- Cell line models of cardiac myocytes are widely regarded as unrepresentative and some are known to lack common cardiac specific markers such as *Nkx2.5*. Primary cultures of
25 mouse cardiac myocytes extracted from 10-12 dpc fetal heart tissue are produced and studied. The effect of *Csl* overexpression is studied in these cells by transfection with expression constructs. Initially, morphological effects and cell proliferation rates are assessed, followed by an examination of specific cardiac marker gene alterations.

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The C2C12 cell line was established from mouse adult leg muscles which were crush-injured to induce satellite cell proliferation (9). These cells maintain a fibroblast-like phenotype in media containing high concentrations of fetal calf serum, but rapidly withdraw from the cell cycle and undergo myogenic differentiation when the media is replaced with lower concentrations of horse serum. These cells express a wide variety of adult muscle-specific markers including the myogenic factors and insulin-like growth factors. In C2C12 cells, which normally expresses Csl at high levels when induced to differentiate, enforced expression of FLAG-Csl had no significant impact on cell morphology during proliferation in high serum, although a major enhancement of cell fusion and/or hypertrophy after serum withdrawal was seen. Western analysis showed that myosin heavy chain levels were increased approximately 10 fold during differentiation, relative to a tubulin control.

Although no discernable phenotype was detected in 10T1/2 fibroblasts constitutively expressing FLAG-Csl protein, constitutive FLAG-Csl expression in L6E9 cells resulted in precocious accumulation of muscle-specific MyHC in myoblasts (Fig. 15), delayed differentiation and increases in MyHC and calcineurin protein levels (Fig. 7,8). The L6E9 parental and L6/Csl lines were analyzed in more detail and many of the markers of differentiation (MyHC, MEF2C) were delayed in their appearance. It is possible that initial association of excess Csl with nascent myosin heavy chains interferes with the formation of muscle sarcomeres. In addition, excess Csl may interfere with normal calcineurin enzymatic activity or other signalling pathways during differentiation.

In L6E9 cells, which express low levels of Csl in the presence of IGF1, enforced expression of FLAG-Csl in L6E9 cells, which also carry a stably integrated transgene expressing IGF1 from a muscle differentiation-specific promoter (the myosin light chain 2 promoter; MLC1 promoter plus MLC1/3 downstream enhancer), had two effects. First, in these cells (L6MLC/IGF-I) Csl induced a massive hypertrophy, as judged by the dramatic increase in the size of myotubes at early differentiation time points. We estimate that Csl-expressing cells form myotubes ("megatubes") that are at least 10 fold

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the diameter of those formed from L6MLC/IGF-I cells alone. Second, even in the presence of high serum, which confers multiple inhibitory signals for myogenic differentiation, FLAG-Csl-expressing L6MLC/IGF-I cells formed myosin-positive multinucleate myotubes at a significant frequency, while L6MLC/IGF-I cells could not
5 differentiate. Under constant positive drug selection for the Csl vector, cultures became completely devoid of Csl-expressing cells within 4 passages. It is thought that the Csl-expressing cells are lost rapidly from the culture due to differentiation. Thus, Csl can override the inhibitory signals for differentiation conferred by high serum and activate the myogenic program. In L6MLC/IGF-I cells expressing FLAG-Csl, myosin heavy
10 chains and the FLAG epitope co-localise, further evidence that Csl can bind myosins.

An antisense *Csl* construct (being an antisense sequence of the full Csl cDNA sequence) in a eukaryotic expression vector is made which is stably integrated into C2C12 cells. Further, an inducible *Csl* expression construct is made because the premature withdrawal
15 from proliferation seen in cotransfected MLC1-IGF and Flag-*Csl* cells has prevented the creation of stable cell lines overexpressing both genes. The *Csl*-induced hypertrophic response in MLC1-IGF L6 cells is also used as a functional bioassay to test, for example, the effectiveness of mutated *Csl* proteins.

20 Transgenic *Csl* mice made with an alpha actin promoter are produced so that the effect of *Csl* overexpression *in-vivo* is observable, particularly with regard to muscle hypertrophy and muscle ageing.

EXAMPLE 10

25 MYOGENIC ACTIVITY OF Csl IN CELL CULTURE ASSAYS

A full molecular workup is performed on the C2C12 and L6MLC/IGF-I cells in which Csl has been over-expressed. Changes in expression of a range of myofilament genes (thick versus thin filament; embryonic versus adult isoforms), are examined as well as
30 those encoding adhesion molecules, cytoskeletal proteins and myogenic regulators of the

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myod and Mef2 families. To avoid loss of differentiating cells during passage in cell culture, the cell lines with Csl expressed from an inducible promoter and/or a differentiation-specific promoter (MLC1 promoter plus MLC1/3 downstream enhancer) are rederived. An antisense Csl vector is constructed to examine the effects of reduce Csl levels on differentiation in culture. The fact that L6E9 and L6MLC/IGF-I cells express Csl poorly or not at all, and that over-expressed Csl enhances differentiation in L6MLC/IGF-I cells, indicates it has an essential function in myogenesis. Cells expressing antisense constructs are monitored for Csl expression and are characterised macroscopically and with the full range of molecular markers.

10

EXAMPLE 11

CSL ENHANCEMENT OF MUSCLE HYPERTROPHY-I

Localized synthesis of IGF-I has been broadly implicated in skeletal muscle growth, hypertrophy and regeneration. The effects of expression of IGF-I on adaptive muscle hypertrophy in L6E9 and C2C12 muscle cell lines is seen morphologically as a significant increase in cell volume and extent of fusion when cells are differentiated (Fig. 7), as well as increases in the enzymes and metabolic end products associated with glycolysis. In hypertrophy associated with high resistance isotonic training in human athletes and in some transgenic models of skeletal muscle hypertrophy, there is a preferential enlargement of fast twitch glycolytic fibers. Hence, the IGF-I model is thought to be directly relevant to these in vivo situations. In L6E9 cells, differentiation and hypertrophy involves the PI3K signal transduction pathway and induction of novel genetic markers.

25

It has been shown that IGF-I-induced skeletal myocyte hypertrophy involves calcineurin-mediated calcium signalling, which also underlies cardiomyocyte hypertrophy (described in detail in Example 12). A novel subset of calcineurin A (CnA) transcripts and nuclear localization of calcineurin protein was induced in a hypertrophic muscle cell line (L6MLC/IGF-I) following post-mitotic activation of a stably transfected IGF-I gene.

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Expression of an activated, calcium-independent CnA mutant in differentiating L6E9 cultures also elicited the hypertrophic response, whereas constitutive expression of a dominant-negative CnA mutant blocked myogenic differentiation. Addition of the calcineurin inhibitor cyclosporin to quiescent L6MLC/IGF-I myoblasts also repressed differentiation, whereas later addition of cyclosporin to fusing myocytes reduced hypertrophy without blocking differentiation. Analyses of transgenic mice expressing the same MLC/IGF-I transcription unit revealed hypertrophic skeletal muscle accompanied by persistent activation of novel CnA gene transcripts in those tissues, indicating that the calcineurin signalling pathway induced by IGF-I in L6E9 cell culture operate in intact skeletal muscle tissue as well.

An analogous model (described in detail in Example 13) of IGF-I-induced skeletal muscle hypertrophy has been established in the C2C12 muscle cell line. Cellular morphometry and analysis of glycolytic status has been carefully quantified, making this a valuable quantitative model. Here, constitutive IGF-I expression induces an increase in calcineurin phosphatase activity from very low baseline levels, presumably in response to Ca^{2+} mobilisation induced by IGF-I receptor activation. Although in this model myogenic differentiation does not depend upon calcineurin, all parameters of hypertrophy could be inhibited with the calcineurin inhibitor, cyclosporin A. Delivery of IGF-I to latissimus dorsi muscles by direct injection of expression plasmid also lead to increased calcineurin phosphatase activity and glycolysis, as well as mobilisation of satellite cells. Thus, these studies define and quantify an IGF-I-induced adaptive hypertrophic response in skeletal muscle cells.

Whereas Csl was not expressed in the parental L6E9 line, a small induction of Csl gene expression in the L6MLC/IGF-I-cell line was observed. No discernible difference in levels of Csl transcripts was detected in the hypertrophic muscles of transgenic mice expressing the MLC/IGF-I-, compared to wildtype muscle (Fig. 5). However, Csl expression was enhanced dramatically in C2C12 cells induced to undergo hypertrophy by addition of IGF-I or insulin and dexamethasone. It is therefore likely that the Csl gene is

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under the direct control of IGF-I-mediated signal transduction pathways leading to hypertrophy. In the transgenic model, steady state with respect to Csl mRNA levels may have been reached in the mice analyzed.

- 5 To test whether Csl might have a synergistic function in IGF-mediated hypertrophy, the FLAG-Csl expression vector was co-transfected with an MLC/IGF-I-expression vector into L6E9 cultures. When the two proteins were co-expressed, a significant synergistic enhancement of the hypertrophic phenotype was achieved upon myogenic differentiation (Fig. 7). While Chisel clearly delays differentiation, as judged by levels of several
- 10 muscle differentiation markers, it has been shown that it enhances the expression of myogenin, a key regulatory gene acting at the onset of differentiation, but it does this only in the context of IGF-I expression (Fig. 16). Even though differentiation is actually delayed in these cultures, increased myogenin clearly demonstrates the interaction between Csl and the IGF-I-induced pathway of differentiation and hypertrophy,
- 15 consistent with the findings described above that enhanced hypertrophy at late stages in the culture is seen only in the context of IGF-I expression (Fig. 7). To dissociate the inhibitor effects of Csl on myocyte differentiation from its synergistic effects on myocyte hypertrophy, the MLC/myc-Csl expression vector, which is activated only after the differentiation program is initiated was used. L6E9 cells transiently co-transfected with
- 20 MLC/IGF-I- x MLC/myc-Csl did not exhibit any delay in differentiation but retained the enhanced hypertrophic phenotype, forming robust hypertrophic cells which could be visualized by anti-myc immunofluorescence. This indicates that the synergistic effects of Csl and IGF-I- can be distinguished from the delay in differentiation, and reinforces the potential biological relevance of the enhanced hypertrophic phenotype.

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EXAMPLE 12
IGF-I INDUCED CALCINEURIN-DEPENDENT
HYPERTROPHY IN SKELETAL MYOCYTES

5 (i) Results

To determine whether calcineurin signalling participates in IGF-I-mediated hypertrophy, the expression pattern of CnA and CnB subunits in both L6E9 and L6MLC/IGF-I cultures were analyzed during differentiation after serum withdrawal (Fig. 17A).

- 10 Transcripts encoding the CnA subunit were undetectable in L6E9 cells under proliferating (GM) or differentiating (DM) culture conditions. Differentiating L6MLC/IGF-I myocytes accumulated abundant CnA transcripts, presumably to increase calcineurin levels in response to IGF-I. One of these transcripts (4.5kb) has been previously described in skeletal muscle (10), whereas a novel transcript (1.7kb) was induced by
- 15 IGF-I expression. Western blot analysis revealed significant amounts of CnA protein in L6E9 myocytes that were further increased in the L6MLC/IGF-I myocytes, (Fig. 17B), suggesting that skeletal muscle cells maintains an intracellular pool of calcineurin. Transcripts encoding CnB were expressed at similar levels in both L6E9 and L6MLC/IGF-I cultures throughout differentiation (Fig. 17A). This indicates that the
- 20 CnB gene is not activated by IGF-I.

Since calcineurin action on targets such as the NF-AT family of transcription factors involves the translocation of the enzyme-substrate complex into the nucleus (11,12) the subcellular localization of calcineurin in L6E9 and L6MLC/IGF-I myocytes was

- 25 analyzed. Immunofluorescence analysis with an anti-CnA antibody (Fig. 17B) revealed that whereas calcineurin was present in both cytoplasm and nuclei of differentiated L6E9 myocyte, characterized by a dramatic increase in size and an unusual organization of nuclei grouped in rings located within the middle of myofibers (373).

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To verify a functional role for calcineurin in the hypertrophic response, lines of L6E9 cells stably transfected with a calcium-independent form of activated CnA (13) under the control of a myogenin promoter were generated. When cultured in DM for only two days, these lines (L6Myog/CnA) displayed a pronounced hypertrophic phenotype (Fig. 18A) typical of L6MLC/IGF-I differentiated cultures (37). Transient transfection of L6MLC/IGF-I myoblasts with a constitutively expressed, dominant negative form of CnA (CnDN-HA (14)) blocked subsequent differentiation, visualized by the mutually exclusive appearance of HA-tagged CnA and muscle myosin (Fig. 18B).

Calcineurin phosphatase activity is strongly inhibited by immunosuppressant-cyclophilin complexes, which recognize specific configurations of the CnB moiety in the calcineurin heterotrimer (15). One of these immunosuppressive drugs, cyclosporin A (CsA), effectively blocks calcineurin activity both in cardiac tissue (16) and in primary skeletal muscle cultures (39). Therefore calcineurin phosphatase activity in differentiating L6E9 and L6MLC/IGF-I cultures with CsA (Fig. 18C) was blocked. Proliferating myoblasts from either cell line treated with CsA were not able to differentiate, as analyzed by MyHC expression.

To determine whether later stages of IGF-I-mediated hypertrophy were also dependent upon the calcineurin pathway, CsA was added to quiescent L6E9 and L6MLC/IGF-I cultures, before or after the myogenic differentiation program was activated, and then analyzed for MyHC expression after five days in DM. Both L6E9 and L6MLC/IGF-I cultures treated with CsA at d0 failed to differentiate (Fig. 18C), as demonstrated by the absence of MyHC protein. The addition of CsA at d2, by which time MLC/IGF-I expression had been activated, did not block MyHC accumulation in either cell line (Fig. 18C), indicating that IGF-I expression may counteract the effects of calcineurin inhibition on the myogenic program through other pathways. Similar results have been obtained by administration of CsA to the myogenic C2C12 cell line, both in hypertrophic transfectants constitutively expressing IGF-I, and in untransfected cultures in which hypertrophy was induced by insulin and dexamethason (17, 46), indicating that

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calcineurin signalling in skeletal muscle effected through exogenous hypertrophic stimuli is also sensitive to CsA. The narrow window of susceptibility to CsA-mediated inhibition of differentiation argues against the general toxicity of the drug, at least at the relatively low concentrations used. Moreover, in L6MLC/IGF-I cultures hypertrophy, but not differentiation, was compromised by CsA added at d2 (Fig. 18C), revealing a specific effect of CsA on the hypertrophic phenotype, and suggesting that continuous calcineurin activity is necessary for the maintenance of IGF-I-induced hypertrophy in this model.

10 Hypertrophy of both skeletal and cardiac muscle depends upon extracellular signals, which are amplified by second messenger molecules and transmitted to the nucleus where they induce changes in gene expression patterns. While the ultimate downstream targets of the hypertrophic response in striated muscle have yet to be identified, calcineurin-mediated hypertrophy in cardiac tissue involves the interaction of the transcription factor NF-AT3, which is activated by calcineurin mediated dephosphorylation, and GATA-4, a factor shown to be critical for proper heart development (18,19). Skeletal myocytes express specific NF-AT isoforms (20), some of which undergo nuclear translocation in the presence of activated calcineurin (21,22). GATA factors have heretofore not been implicated in skeletal muscle gene regulation. However in C2C12 skeletal muscle cultures, GATA-2 transcripts are present in myoblasts and are reduced during differentiation, whereas GATA-2 protein is preferentially accumulated in differentiated myocytes.

To establish a potential role for GATA factors in IGF-I-mediated skeletal muscle hypertrophy, the pattern of GATA-2 gene activation in L6E9 and L6MLC/IGF-I cultures was analyzed with Northern blot analysis and immunofluorescence (Fig. 19). GATA-2 transcripts were undetectable in L6E9 proliferating and differentiated myoblasts, but were already present in L6MLC/IGF-I cultures after one day in DM, increasing on subsequent days (Fig. 19A). Addition of CsA to L6MLC/IGF-I quiescent myoblasts reduced subsequent GATA-2 activation in DM (Fig. 19B, lane 2 vs. 3). Consistent with this

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result, a low but significant increase in GATA-2 transcripts was seen in the L6E9 lines stably transfected with an activated calcineurin expression vector (L6Myog/CnA; Fig. 19B, compare lane 1 and 4).

5 As predicted from its transcript distribution, GATA-2 protein was not detected by immunofluorescence analysis in L6E9 or L6MLC/IGF-I proliferative myoblasts, nor in differentiated L6E9 cultures (Fig. 19C left). In contrast, GATA-2 protein was abundant and localized to the nuclei in L6MLC/IGF-I hypertrophic myofibers, but absent from non-hypertrophic myocytes in less densely seeded regions of the same culture (Fig. 19C
10 right).

To verify whether IGF-I mediated GATA-2 activation is a consequence of calcineurin signalling, or involves an alternative pathway, the subcellular localization of GATA-2 protein was localized to the nuclear rings in the L6Myog/CnA hypertrophic myocytes
15 (Fig. 19D), as seen in L6MLC/IGF-I cultures. The induction of GATA-2 exclusively in hypertrophic nuclei of L6MLC/IGF-I and L6Myog/CnA cultures underscores its potential role in the orchestration of the hypertrophic gene program, and defines it as a novel marker of the hypertrophic response in skeletal muscle cells.

20 These results also confirm that localized GATA-2 expression can be activated by calcineurin in hypertrophic nuclei.

(ii) Materials and Methods

25 Cell culture and transfection

The myogenic parental cell line L6E9 (23,24) and the stable clone L6MLC/IGF-I (13) were maintained in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% Fetal Bovine Serum (FBS). Cultures were
30 plated at 6×10^5 cells/100 mm dish for RNA or protein extraction. For stable

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transfection, L6E9 cells were grown in 100 mm plates to 70% confluence and then washed with serum- and antibiotic-free medium. Cells were transfected with a calcium-independent form of activated Calcineurin-A under the control of myogenin promoter (Myo 1565) and with puromycin-selectable-vector (pPUR, Clontech) (10:1), as
5 previously described (13). Stable myoblasts were maintained in DMEM selection medium (20% FBS, 3 µg/ml puromycin), which was replaced every two days, for 12 days. Twelve drug-resistant clones (L6Myog/CnA) were isolated using cloning rings and characterized morphologically, and by Northern blot analysis for CnA expression. The morphology was equivalent between high and low expressing L6Myog/CnA clones.

10

For transient transfections, L6MLC/IGF-I cells were transfected with a calcineurin-dominant negative-HA-tagged plasmid (CnDN-HA) (14). To activate myogenic differentiation myoblasts were switched to differentiation medium (DM) (DMEM + 1%BSA), and analyzed by double immunofluorescence or CnDN-HA and sarcomeric
15 myosin expression, after 2 days in DM.

Cyclosporin-A (CsA) treatment

Cells were grown as described above and treated with CsA, an inhibitor of the
20 Calcineurin phosphatase activity. The cultures were treated with 3 µM or 5 µM CsA in ethanol, as outlined in the text, at different times in the differentiation program: growth medium (GM), 80% confluence (day 0), and day 2 (d2) in DM. Cells treated at d0 or d2 were continuously exposed to CsA until they were harvested at d5. Cells treated during GM with CsA in ethanol were switched to DM without CnA inhibitor when they were at
25 80% confluence. Cells that did not receive inhibitor received ethanol as control vehicle. At d5 cells were analyzed by Northern or immunofluorescence analysis.

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RNA preparation and Northern blot analysis

Total RNA from L6E9, L6MLC/IGF-I, and L6Myog/CnA cultures was obtained by RNA-TRIZOL extraction (Gibco-BRL). Fifteen μ g of total RNA were fractionated by electrophoresis on 1.3% agarose gels containing 2.2 M formaldehyde, transferred directly from the gel to Nytran membranes (Gene Screen Plus, NEN), and hybridized as described previously (13). cDNA probes were 32 P labelled by random priming.

Immunofluorescence analysis

10

L6E9, L6MLC/IGF-I, and L6Myog/CnA differentiated cultures were fixed in 4% paraformaldehyde and processed as previously described (13). Nuclei of myogenic cells were visualized using Hoechst staining. The following antibodies were used: a monoclonal MF-20 antibody (Hybridoma Bank), a monoclonal antibody to calcineurin (Signal Transduction Laboratory); a monoclonal antibody to GATA-2 (Santa Cruz); and a rabbit polyclonal to HA (Santa Cruz).

Western blot analysis

20 L6E9 and L6MLC/IGF-I differentiated cultures were washed with ice-cold PBS followed by lysis in 0.5 ml of Lysis Buffer 25 mM Hepes pH 7.2, 100 mM NaCl, 2 mM MgCl_2 , 0.5% Triton X-100, 1 mM PMSF, and 1 μ g/ml each of Leupeptin, Antipain, Aprotinin, and Pepstatin (25). Proteins were separated by 12% polyacrylamide gel (SDS-PAGE), probed with calcineurin antibody (1:150), and developed using ECL reagents (Amersham).

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EXAMPLE 13
SKELETAL MUSCLE HYPERTROPHY MEDIATION BY A
CALCINEURIN-DEPENDENT SIGNALLING PATHWAY

5 (i) **Results**

IGF-I mediates growth of muscle and other tissues, and in cultured myoblasts is both a potent proliferative and differentiating agent acting through the MAP-kinase and PI-3-kinase pathways (26), respectively. To test if IGF-I can also produce myotube

10 hypertrophy, the C2C12 myoblast cell line, derived from adult mouse satellite cells, was stably transfected with a plasmid encoding IGF-I (27). Clonal cell lines thus derived secreted IGF-I into the culture medium and, after differentiation of the cells in low serum medium, resulted in myotube hypertrophy (Fig. 20a). Hypertrophy was evident by an increase in myotube size (mean area, Fig. 20b, and width, data not shown), and by an

15 increase in protein synthesis in the absence of increased DNA replication ($[^3\text{H}]$ -thymidine uptake) (Fig. 20c). Selective enlargement of fast-twitch glycolytic myofibers is observed with high-resistance isotonic exercise in humans (28), and in some transgenic mouse models of skeletal muscle hypertrophy (29). Conversely, this fiber-type is selectively lost with ageing (30) and in animals models of muscular dystrophy (31). Thus the

20 metabolic status of IGF-I myotubes was evaluated and it was observed that both the activity of glycolytic enzymes and the end-product of anaerobic metabolism, lactate, were significantly increased in IGF-I myotubes (Fig. 20b). Since IGF-I-transfected C2C12 cells were clonal, this indicates that IGF-I not only induces muscle hypertrophy, but can mediate an adaptive, developmental switch in myofiber phenotype.

25

It has recently been shown that the Ca^{2+} /calmodulin-dependent phosphatase, calcineurin, known to mediate activation of B and T lymphocytes, also functions in the induction of cardiac hypertrophy (32). In some (32) but not all models (33,34), cardiac hypertrophy can be prevented by the calcineurin inhibitors, cyclosporin A (CsA) and FK-506. As

30 shown in Figure 2, stead-state levels of the 60kDa catalytic subunit (CnA) and 19kDa

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regulatory subunit (CnB) of calcineurin were unaltered in IGF-I myotubes. However, compared to control C2C12 cells transfected with vector only, calcineurin phosphatase activity was increased in IGF-I myoblasts and remained high during differentiation to myotubes (Fig. 21). In the *in vitro* phosphatase assay, calcineurin activity in IGF-I

5 transfected cells was high in the absence of exogenously added calmodulin and remained unchanged with increasing concentrations (Fig. 22b). This indicates that with IGF-I-induced myotube hypertrophy, calcineurin is already maximally activated. In the absence of changes in CnA or CnB expression, increased calcineurin activity is thought to be due to IGF-I-induced Ca^{2+} -mobilisation (35).

10

To evaluate if calcineurin activation was causally related to IGF-I-induced myotube hypertrophy, cells were treated with the calcineurin inhibitor, CsA. As shown in Figure 20, CsA (1 μM) prevented all parameters of myotube hypertrophy, including the IGF-I-induced switch to anaerobic glycolysis. CsA (0.5-1.0 μM) had no effect on IGF-I-

15 induced differentiation. However, CsA inhibited IGF-I-induced proliferation ($[\text{}^3\text{H}]$ -thymidine uptake) of transfected myoblasts by 91 % at 1 μM , and 95 % at 3 μM , but not proliferation of control myoblasts (< 1 % at 3 μM). Inhibition of IGF-I-induced proliferation by CsA appears unrelated to its effects of myotube hypertrophy, since inhibition of the MAP kinase pathway with PD98059 (a MEK-1 inhibitor) also

20 suppressed IGF-I-induced proliferation (by 93 % at 100 μM), but did not prevent myotube hypertrophy or the switch to anaerobic metabolism. Inhibitors of the PI-3-kinase pathway (LY294002, a PI-3-kinase inhibitor, a rapamycin, a PI-3-kinase pathway inhibitor acting downstream via mTOR to inhibit p70^{S6K}), at doses (10 μM and 1 $\mu\text{g/ml}$, respectively, added during the myoblast phase) that completely prevent differentiation of L6

25 myoblasts (36,37), had no effect on either IGF-I-induced hypertrophic or proliferative responses.

To confirm that involvement of the calcineurin pathway is not unique to the IGF-I model, C2C12 cells were treated with insulin (I, 1 μM) and dexamethasone (D, 2.5 μM) from

30 the time cells were switch to low serum differentiation medium. Dexamethasone

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augments myogenic differentiation in C2C12 cells, potentially by enhancing and modifying IGF-I and/or insulin receptor tyrosine kinase-mediated signalling (38). Like IGF-I, I/D treatment induced myotube hypertrophy, a switch to anaerobic metabolism and activation of calcineurin, and all responses could be prevented by CsA (Fig. 22).

- 5 The timing of IGF-I-induced myotube hypertrophy and its inhibition by CsA was investigated in more detail.

CsA inhibited myotube hypertrophy of IGF-I-transfected cells only if added at the myoblast stage, but not post-differentiation, and only if added to I/D-treated cells before
10 or at the time of differentiation, but not after. These results identify a critical window for induction of hypertrophy that lies early in the transition from myoblast to post-mitotic myocyte. This is consistent with the contention that maintenance of type IIb fibres in IGF-I/AAV (adeno- associated virus)-treated muscle (39), and generation of hypertrophy in muscles subjected to chronic work-overload (40), involves fusion of activated satellite
15 cells with, rather than direct effects on, existing myofibers.

Finally, the *in vivo* effects of IGF-I were evaluated by direct injection of a plasmid encoding IGF-I into latissimus dorsi muscle (LDM) of rats; the contralateral LDM, which received vector only, served as control. In the IGF-I-injected LDM,
20 transformation was observed in approximately 10% of skeletal muscle cells in the immediate vicinity (2 mm radius) of the injection sites, as seen by coexpressed green fluorescent protein, and expression of IGF-I mRNA persisted for at least three months (Fig. 23a,b). When harvested two months after IGF-I injection, LDM showed not only increased calcineurin activity and lactate levels (Fig. 23b,c), but also an increased
25 number of myofibers with central nuclei (Fig. 23d), a hallmark of new muscle formation due to satellite cell activation (39).

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(ii) Materials and Methods

Plasmid Construction.

- 5 A plasmid (pIGF-1/IRES/GFP20) based on pcDNA3 and allowing stable transfection and expression of IGF-1 under the control of an elongation factor-1 α promoter, was prepared as described (5). The construct contained an internal ribosome entry site and cDNA for the green fluorescent protein variant, GFP20, downstream of the IGF-1 cDNA, allowing coexpression of GFP20. The construct also contained the neomycin resistance gene. A
10 control vector was identical but lacked the IGF-1 cDNA.

Cell lines, transfection and culture.

- C2C12 cells were transfected with pIGF-1/IRES/GFP20 and clonal lines established as
15 described (5). Stably transfected clones were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% foetal calf serum and Geneticin (G, 200 μ g/mL). To induce differentiation, the medium was changed to DMEM plus 2% horse serum and G (200 μ g/mL). GFP20 fluorescence was assessed by epifluorescence using a fluorescein isothiocyanate filter (Zeiss Oxiolab). Cells were grown in the absence or presence of
20 cyclosporin A, LY294002 (Sigma), rapamycin (ICN Laboratories) or PD98059 (New England Biolabs), as indicated.

Evaluation of protein and DNA synthesis and anaerobic glycolysis.

- 25 Fractional protein synthesis rate (L-[2,6- 3 H]-phenylalanine (Amersham) uptake), DNA synthesis ([3 H] thymidine (Amersham) incorporation), and anaerobic glycolysis (lactate dehydrogenase and alanine aminotransferase activities, and lactate levels) were determined as described (27).

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Histological analysis.

Myotube tube morphology was determined after fixation in 10% methanol, and staining with 5% Giemsa. Myotube area and width were determined by quantitative morphometry using the Leica Q500MC image analysis software, as described⁵. For each treatment group, 100 myotubes were analysed from five randomly selected high-power (X40 magnification) fields.

Calcineurin phosphate (CnPP) activity.

10

C2C12 cells were washing in PBS and lysed by freeze/thawing in 50 µl of buffer containing 50mM Tris, pH 7.5, 0.1mM EGTA, 1mM EDTA, 0.5mM dithiothreitol, 0.5mM phenylmethylsulfonylfluoride, and 5 µg of each of leupeptin, aprotinin, and soybean trypsin inhibitor. Extracts were cleared by centrifugation at 10,000xg for 10 minutes at 4°C. CnPP activity in resulting supernatant fractions was determined in the presence of 0.1mM Ca²⁺ and 100nM calmodulin (except in the experiments indicated, where calmodulin was varied from 0 to 100nM), as described by Fruman *et al.* (41), and calculated from the difference in [³²P] released in the absence and presence of a calcineurin-specific inhibitor peptide (peptide 412) (41,42).

20

Immunoblot analysis for calcineurin A (CnA) and B (CnB) subunits.

Whole cell extracts were prepared from C2C12 cells by the addition of 200 µl of lysis buffer (2% sodium dodecyl sulfate (SDS), 10mM Tris, Hcl, pH 7.4) per 10cm dish after three initial washes with PBS. After boiling for 5 minutes, the supernatant were fractionated by SDS-polyacrylamide gel electrophoresis (8% for CnA; 12% for CnB). Immunoblotting was performed using standard procedures (43) with an anti-CnA (1:3000 final dilution) or anti-CnB (1:2000) primary antibody, followed by a sheep anti-mouse HRP-tagged secondary antibody (1:1000; Amersham), and resolution by ECL chemiluminescence (New England Life Science).

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In vivo IGF-1 transformation.

Rats (Wistar, male, n=8) were anaesthetised with 5% halothane and the latissimus dorsi muscles (LDMs) exposed by a midline incision. pIGF-1/IRES/GFP20 (100 µg) was
5 injected into one LDM, and the control vector (100 µg) into the contralateral LDM, using a 25 gauge needle and 20 injections/cm² of muscle. The incision was then closed with surgical skin clips and after recovery the animals maintained on standard rat chow and water. At the times indicated, animals were sacrificed and LDM harvested for RNA
10 extraction and Northern blot analysis (27), calcineurin phosphatase activity, and histology. All studies were performed in accordance with NH&MRC guidelines and approval of the St. Vincent's Hospital Animal Ethics Committee.

Data analysis.

15 Comparisons between treatments were made using paired or unpaired *t* tests, or χ^2 analysis, with $p < 0.05$ being considered statistically significant. All data are expressed as the mean \pm 1 S.E.M (error bars).

EXAMPLE 14**20 CREATION OF A NULL MUTATION OF *Csl***

The X chromosomal location of the gene complicates the issue since the embryonic stem (ES) cell line used to make knockouts is XY and is therefore hemizygous for *Csl*. If a null mutation is introduced into the single copy of *Csl*, all *Csl* expression is lost.
25 Assuming that lack of *Csl* expression is significantly deleterious, chimeras made from the targeted cell line mixed with wild-type blastocysts may not survive if the proportion of the targeted cells is very high as it needs to be to achieve germ line transmission. Under these circumstances the effect of the knockout is studied in fetal chimeras.

30

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A conditional knockout construct has been designed that is intended to maintain unaltered *Csl* expression in the primary targeted conformation and allows targeting of the *Csl* gene deletion exclusively to skeletal or cardiac muscle. The introduction of loxP sites from the coliphage P1 system (44) allows the later excision of a portion of this construct on exposure to Cre recombinase. The construct is built to replace the second exon which contains a short stretch of 5' untranslated region, the initiating methionine codon and the amino terminus of the predicted protein which by comparison with human and *Xenopus* sequences appears to be the most conserved domain. The construct does not remove any exon or intron sequence but introduces one loxP site within the 5' untranslated region and another within the intron between exon 2 and 3. A PGK-neo selection cassette has been introduced into the intron between the two loxP sites and a human placental alkaline phosphatase cDNA has been placed after the intronic loxP site. Cre recombination excises the intervening sequence between the two loxP sites thus removing the selection cassette, the 5' portion of *Csl* coding sequence and the splice donor of exon 2. The coding sequence of alkaline phosphatase is brought into the spliced transcript in a position previously occupied by the initiation codon of *Csl* and if a stable mRNA is formed which is translated it allows the histochemical detection of alkaline phosphatase in cells that have undergone Cre excision. In any event, *Csl* translation is destroyed.

A knockout allele of *Csl* using elements of Cre/Loxp is depicted in Fig. 24. The construct is designed not to disrupt expression or splicing of the *Csl* gene until exposure to Cre recombinase, which would then remove the initiation codon and the protein coding region of exon 2, thus inactivating the allele. A marker gene, human placental alkaline phosphatase (HPAP), is designed to be expressed from the *Csl* promoter after excision with Cre, thereby allowing direct visualization of cells with deleted alleles. The construct has been electroporated into ES cells and numerous targeted clones identified and isolated (Fig. 25). After validation of the structure of the targeted allele with other probes, these are injected into blastocysts for creation of knockout mice.

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The mice are bred to females to produce Csl-targeted (*Csl*^f) hemizygous males and *Csl*^f heterozygous females which are used to maintain the line. To produce null mutants, male hemizygous *Csl*^f mice or the founder chimeras are mated to transgenic females expressing Cre in the germ line (45). Since this Cre transgene is inserted on the X

5 chromosome it is necessary to breed a second generation from the femal progeny of this cross because only the females carry both the *Csl*^f allele and the Cre transgene. Males produced from this second generation all carry the null mutation whereas the females are heterozygous null.

- 10 Phenotypes of both heterozygote (female) and homozygote (male) offspring are analyzed for cardiac and skeletal muscle defects. If deletion of one or both *Csl* alleles proves to be lethal, conditional knockout strategies described above will be invoked to dissect in detail the effects of *Csl* deficiency during development.
- 15 Alternatively, the *Csl* knockout is activated in a tissue-specific fashion by breeding *Csl*^f mice with lines that express the Cre protein under the control of promoters that are active in the embryonic heart or skeletal muscle.

EXAMPLE 15

20 MODULATION OF CALCINEURIN SIGNALLING IN HEART AND SKELETAL MUSCLE

Csl plays a role in calcineurin-mediated signalling through direct interaction with the CnA catalytic subunit. The composition of active calcineurin, as analyzed historically in

25 T lymphocytes and neurons, typically comprises a heterotrimer between the CnA catalytic and CnB regulatory subunits with Ca²⁺-activated calmodulin. The importance of the calcineurin pathway in tissues other than the immune and nervous systems has only recently begun to be recognized. Alternate isoforms of calcineurin subunits could preferentially incorporate *Csl* as an alternate regulatory partner. *Csl* could shift the

30 substrate specificity of calcineurin enzymatic activity in striated muscle under various

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conditions, in which case Csl would play a key role in the beneficial or detrimental effects of calcineurin signalling in these tissues.

EXAMPLE 16

DRUG DISCOVERY

5

The intense focus during the last decade on compounds which act as immunosuppressants by blocking calcineurin signalling in T lymphocytes underscores the potential clinical importance of a novel target for drugs to mediate this critical pathway. The currently
10 available pharmacological inhibitors of calcineurin phosphatase, cyclosporin A and FK506, are directed against the regulatory CnB subunit in the calcineurin heterotrimer responsible for T lymphocyte activation. Although these drugs are in wide use, their adverse side effects, such as renal impairment, hypertension and metastatic cancer, are serious.

15

A novel application of these drugs has been recently proposed in the modulation of human hypertrophic cardiomyopathies, since cardiac hypertrophy can be experimentally induced by expression in transgenic mouse myocardium of an activated form of calcineurin. Furthermore, hypertrophy associated with this and some other transgenic
20 mouse model can be temporarily blocked by administration of cyclosporin A or FK506. It seems clear, however, from additional animal studies that these compounds will not be useful in all hypertrophy situations, particularly that associated with pressure overload induced by hypertension. Csl, due to its cardiac and skeletal muscle specificity of expression, comprises a more directed target for pharmacological intervention in
25 calcineurin signalling. In the heart, this could be either to abrogate undesirable hypertrophy in the case of hypertrophic cardiomyopathies, or to enhance compensatory hypertrophy in the case of heart failure due to infarction and/or dilated cardiomyopathy. In skeletal muscle, it could be to enhance myogenic adaption and regeneration during ageing or in myopathies as detailed above.

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EXAMPLE 17

GENE MUTATIONS

As a potential regulatory molecule mapping to the X chromosome, Csl represents a
5 candidate for X-linked genetic disorders affecting heart and skeletal muscle. Screens for
polymorphisms in the Csl locus within populations carrying unmapped X-linked diseases
would be highly informative.

EXAMPLE 18

GENE THERAPY

10

Progress in gene therapy for skeletal muscle pathologies has been slow, partially because
many of the genes to be introduced encode extremely large proteins that are difficult to
engineer into vectors. In contrast, the relatively small size of the Csl protein makes it
15 highly tractable as a gene therapeutic agent. In the heart, the potential reversal of
cardiomyopathic syndromes by Csl gene introduction into postmitotic cells, inducing
compensatory hypertrophy or realignment of cardiomyocytes, represents an important
advance in treatment of these common and often lethal pathologies. If warranted by the
emerging biology of Csl, abnormal muscle cell function, the gene could be readily and
20 permanently delivered with AAV-based viral technologies, to a variety of postmitotic
heart and skeletal muscle targets.

Those skilled in the art will appreciate that the invention described herein is susceptible
to variations and modifications other than those specifically described. It is to be
25 understood that the invention includes all such variations and modifications. The
invention also includes all of the steps, features, compositions and compounds referred to
or indicated in this specification, individually or collectively, and any and all
combinations of any two or more of said steps or features.

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TABLE 2:**ESTs with significant homology to mouse chisel.**

5	gb AA033164 AA033164 gb AA434782 AA434782 gb AI035961 AI035961 gb W36988 W36988 gb AA060214 AA060214
10	gb W18392 W18392 gb AA499581 AA499581 gb W13738 W13738 gb W29828 W29828 gb AI098485 AI098485
15	gb AI153970 AI153970 gb W97451 W97451 gb W29186 W29186 gb AA763276 AA763276 gb W18646 W18646
20	gb W14689 W14689 gb AA211521 AA211521 gb W07478 W07478 gb AA511812 AA511812 gb AA389647 AA389647
25	gb AA248485 AA248485 gb R58129 R58129 gb N86813 N86813 gb AA211443 AA211443 gb AI090520 AI090520
30	gb N87511 N87511

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gblAI333485IAI333485
 gblAI355676IAI355676
 gblAI190689IAI190689
 gblAA092554IAA092554
 5 gblAA214155IAA214155
 gblAA094015IAA094015
 gblN56276IN56276
 emblF16837IHSPD01347
 gblN75838IN75838
 10 gblN55839IN55839
 gblAI381720IAI381720
 gblAA214147IAA214157
 gblAA247862IAA247862
 gblN84085IN84085
 15 gblAA214031IAA214031
 gblAA248067IAA248067
 gblAA867785IA867785

Table 2. Current listing of mouse and human expressed sequence tags (ESTs) with
 20 highly significant homology to the mouse *Csl* cDNA sequence. This list was obtained
 using the NCBI BLAST program. Several highly significant rat ESTs are also identified
 by the BLAST search which have been excluded. The figures correspond to the EMBL
 and GenBank accession numbers.

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CLAIMS:

1. An isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or a complementary to a nucleotide sequence encoding, a protein or derivative or homolog thereof wherein said sequence is expressed in heart muscles or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleic acid molecule.
2. An isolated nucleic acid molecule according to claim 1 wherein said protein comprises the amino acid sequence substantially as set forth in SEQ ID NO:2 or a derivative, homolog or mimetic thereof or having at least about 45% or greater similarity to SEQ ID NO:2 or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleic acid molecule.
3. An isolated nucleic acid molecule according to claim 1 comprising a nucleotide sequence substantially as set forth in SEQ ID NO:1 or a derivative, homolog or mimetic thereof or capable of hybridizing to SEQ ID NO:1 under low stringency conditions or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleic acid molecule.
4. An isolated nucleic acid molecule according to claim 3 which further encodes an amino acid sequence corresponding to an amino acid sequence substantially as set forth in SEQ ID NO:2 or a sequence having at least about 45% similarity to SEQ ID NO:2.
5. An isolated nucleic acid molecule according to claim 2 or 3 substantially as set forth in SEQ ID NO:1.
6. An isolated nucleic acid molecule according to claim 1 wherein said protein has the characteristics of Csl or a functional equivalent thereof.

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7. An isolated nucleic acid molecule according to claim 1 wherein said protein comprises the amino acid sequence substantially as set forth in SEQ ID NO:4 or a derivative, homolog or mimetic thereof having at least about 45% or greater similarity to SEQ ID NO:4 or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleic acid molecule.
8. An isolated nucleic acid molecule according to claim 1 comprising a nucleotide sequence substantially as set forth in SEQ ID NO:3 or a derivative, homolog or mimetic thereof or capable of hybridising to SEQ ID NO:3 under low stringency conditions or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleic acid molecule.
9. An isolated nucleic acid molecule according to claim 8 which further encodes an amino acid sequence corresponding to an amino acid sequence substantially as set forth in SEQ ID NO:4 or a sequence having at least about 45% similarity to SEQ ID NO:4.
10. An isolated nucleic acid molecule according to claim 7 or 8 substantially as set forth in SEQ ID NO:3.
11. An isolated nucleic acid molecule according to claim 1 wherein said protein comprises the amino acid sequence substantially as set forth in SEQ ID NO:5 or a derivative, homolog or mimetic thereof or having at least about 45% or greater similarity to SEQ ID NO:5 or a derivative, homolog, analog, chemical equivalent or mimetic of said nucleic acid molecule.
12. An isolated nucleic acid molecule according to claim 1 comprising a nucleotide sequence comprising exon regions of which five comprise

Exon 1 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:6;

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Exon 5 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO: 10,

16. An isolated protein according to claim 15 comprising an amino acid sequence substantially as set forth in SEQ ID NO:2 or a derivative, homolog or mimetic thereof or having at least about 45% or greater similarity to SEQ ID NO:2 or a derivative, homolog, analog, chemical equivalent or mimetic of said protein.

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17. An isolated protein according to claim 15 or 16 encoded by a nucleotide sequence substantially as set forth in SEQ ID NO:1 or a derivative, homolog or mimetic thereof or capable of hybridizing to SEQ ID NO:1 under low stringency conditions or a derivative, homolog, analog, chemical equivalent or mimetic of said protein.

18. An isolated protein according to claim 16 or 17 substantially as set forth in SEQ ID NO:2.

19. An isolated protein according to claim 15 wherein said protein has the characteristics of Csl or a functional equivalent thereof.

20. An isolated protein according to claim 15 comprising an amino acid sequence substantially as set forth in SEQ ID NO:4 or a derivative, homolog or mimetic thereof or having at least about 45% or greater similarity to SEQ ID NO:4 or a derivative, homolog, analog, chemical equivalent or mimetic of said protein.

21. An isolated protein according to claim 15 or 20 encoded by a nucleotide sequence substantially as set forth in SEQ ID NO:3 or a derivative, homolog or mimetic thereof or capable of hybridizing to SEQ ID NO:3 under low stringency conditions or a derivative, homolog, analog, chemical equivalent or mimetic of said protein.

22. An isolated protein according to claim 15 or 20 encoded by a nucleotide sequence comprising exon regions of which five comprise

Exon 1 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:6;

Exon 2 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:7;

Exon 3 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:8;

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Exon 4 comprising the nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:9; and

Exon 5 comprising a nucleotide sequence corresponding to the nucleotide sequence substantially as set forth in SEQ ID NO:10,

or capable of hybridizing to a genomic sequence comprising said exon regions under low stringency conditions or a derivative, homolog, analog, chemical equivalent or mimetic of said protein.

23. An isolated protein according to claim 22 wherein said nucleotide sequence corresponds to the gene map as set forth in Figure 2.

24. An isolated protein according to claim 20, 21, 22 or 23 substantially as set forth in SEQ ID NO:4.

25. An isolated protein according to claim 15 comprising an amino acid sequence substantially as set forth in SEQ ID NO:5 or a derivative, homolog or mimetic thereof or having at least about 45% or greater similarity to SEQ ID NO:5 or a derivative, homolog, analog, chemical equivalent or mimetic of said protein.

26. An isolated protein according to any one of claims 15-25 which protein is a homodimer.

27. An isolated protein according to any one of claims 15-25 which protein is a heterodimer.

28. A method for modulating expression of *Csl* in a mammal, said method comprising contacting the *Csl* gene with an effective amount of an agent for a time and under conditions sufficient to modulate expression of *Csl*.

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29. A method for modulating the functional activity of Csl in a mammal, said method comprising administering to said mammal a modulating effective amount of an agent for a time and under conditions sufficient to increase or decrease Csl activity.

30. A method for modulating muscle cell functional activity in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a nucleotide sequence encoding Csl or sufficient to modulate the activity of Csl.

31. A method of modulating muscle cell functional activity in a mammal said method comprising administering to said mammal an effective amount of a protein according to any one of claims 15-25 or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate the functional activity of said muscle cell.

32. A method according to claim 31 wherein said protein interacts, binds or otherwise associates with an EF-Hand target.

33. A method according to claim 32 wherein said EF-Hand target is the IQ site on the myosin heavy chain or a calcineurin-A catalytic subunit.

34. A method of modulating muscle cell functional activity in a mammal said method comprising administering to said mammal an effective amount of a nucleic acid molecule according to any one of claims 1-14 or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate the functional activity of said muscle cell.

35. A method according to claim 34 wherein the expression product of said nucleic acid molecule interacts, binds or otherwise associates with an EF-Hand target.

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36. A method according to claim 35 wherein said EF-Hand target is the IQ site on a myosin heavy chain or a calcineurin-A catalytic subunit.

37. A method of modulating cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of a protein according to any one of claims 15-25 or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate the activity of one or more components of a calcineurin-dependent signalling pathway.

38. A method according to claim 37 wherein said cellular functional activity is muscle cell functional activity.

39. A method according to claim 37 or 38 wherein said component is the calcineurin-A catalytic subunit.

40. A method of modulating cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of a nucleic acid molecule according to any one of claims 1-14 or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate the activity of one or more components of a calcineurin-dependent signalling pathway.

41. A method according to claim 40 wherein said cellular functional activity is muscle cell functional activity.

42. A method according to claim 40 or 41 wherein said component is the calcineurin-A catalytic subunit.

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43. A method of modulating cellular functional activity in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a nucleotide sequence encoding *Csl* or sufficient to modulate the activity of *Csl* wherein said *Csl* expression product or *Csl* modulates the activity of one or more components of a calcineurin-dependent signalling pathway.
44. A method according to claim 43 wherein said cellular functional activity is muscle cell activity.
45. A method according to claim 43 or 44 wherein said component is the calcineurin-A catalytic subunit.
46. A method of treating a mammal said method comprising administering to said mammal an effective amount of an agent capable of modulating the expression of *Csl* for a time and under conditions sufficient to modulate muscle cell functional activity.
47. A method of treating a mammal said method comprising administering to said mammal an effective amount of an agent capable of modulating the activity of *Csl* for a time and under conditions sufficient to modulate muscle cell functional activity.
48. A method of treating a mammal said method comprising administering to said mammal an effective amount of a protein according to any one of claims 15-25 or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate muscle cell functional activity.
49. A method of treating a mammal said method comprising administering to said mammal an effective amount of a nucleic acid molecule according to any one of claims 1-14 or a derivative, homolog, analog, chemical equivalent or mimetic thereof for a time and under conditions sufficient to modulate muscle cell functional activity.

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50. A method of treating a mammal according to any one of claims 46 to 49 wherein said mammal is suffering from diabetic cardiomyopathy, cardiac hypertrophy (acquired or familial), heart failure, dilated cardiomyopathy, myocarditis, loss of myofibers and loss of regenerative capacity in ageing and skeletal myopathies such as Duchenne muscular dystrophy and Becker's myotonic dystrophy or myofiber atrophy.

51. A method according to any one of claims 46 to 50 wherein said mammal is administered with a combination of Csl and IGF-I or derivative, homolog, analog, chemical equivalent or mimetic of said Csl and/or IGF-I.

52. Use of an agent capable of modulating the expression of *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof in the manufacture of a medicament for the modulation of muscle cell functional activity.

53. Use of an agent capable of modulating the activity of Csl or a derivative, homolog, analog, chemical equivalent or mimetic thereof in the manufacture of a medicament for the modulation of muscle cell functional activity.

54. Use of Csl or *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof in the manufacture of a medicament for the modulation of muscle functional activity.

55. An agent for use in modulating Csl or a derivative, homolog, analog, chemical equivalent or mimetic thereof wherein modulating said Csl modulates muscle cell functional activity.

56. An agent for use in modulating *Csl* expression or a derivative, homolog, analog, chemical equivalent or mimetic thereof wherein modulating expression of said *Csl* modulates muscle cell functional activity.

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57. Csl or *Csl* or a derivative, homolog, analog, chemical equivalent or mimetic thereof for use in modulating muscle cell functional activity.
58. A pharmaceutical composition comprising *Csl*, Csl or an agent capable of modulating *Csl* expression or Csl activity or derivative, homolog, analog, chemical equivalent or mimetic thereof together with one or more pharmaceutically acceptable carriers and/or diluents.
59. An isolated antibody directed to the protein according to any one of claims 15-25.
60. An isolated antibody directed to the nucleic acid molecule according to any one of claims 1-14.
61. The antibody according to claim 59 or 60 wherein said antibody is a monoclonal antibodies.
62. The antibody according to claim 59 or 60 wherein said antibody is a polyclonal antibody.
63. A method for detecting Csl in a biological sample said method comprising contacting said biological sample with an antibody specific for Csl or its derivatives, homologs, analog, chemical equivalents or mimetics thereof for a time and conditions sufficient for an antibody-Csl complex to form and then detecting said complex.
64. A method for detecting *Csl* in a biological sample said method comprising contacting said biological sample with an antibody specific for *Csl* or its derivatives, homologs, analogs, chemical equivalents or mimetics thereof for a time and conditions sufficient for an antibody-*Csl* complex to form and then detecting said complex.

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65. A method of diagnosing or monitoring a mammalian disease condition, which disease condition is characterized by aberrant muscle cell functional activity, said method comprising screening for *Csl* or *Csl* in a biological sample isolated from said mammal.

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FIG 1

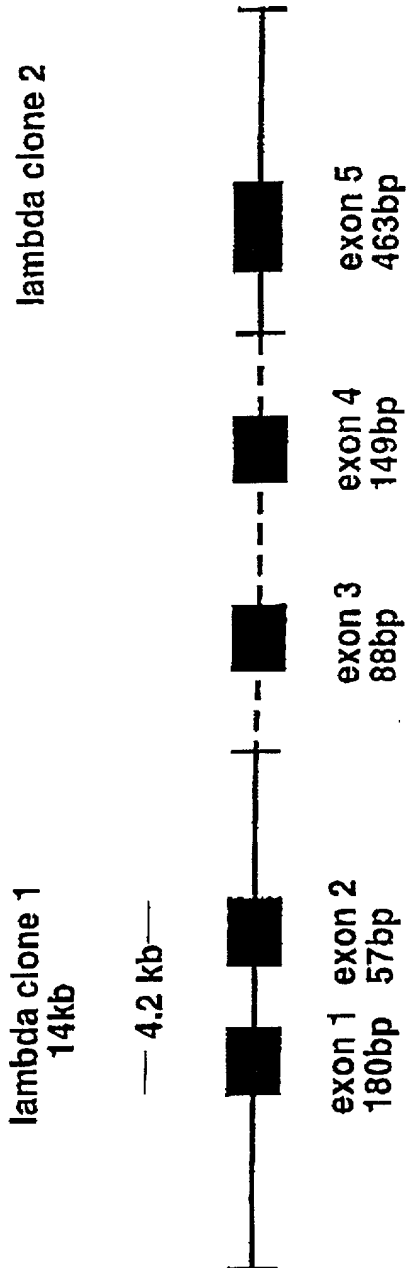


FIGURE 1

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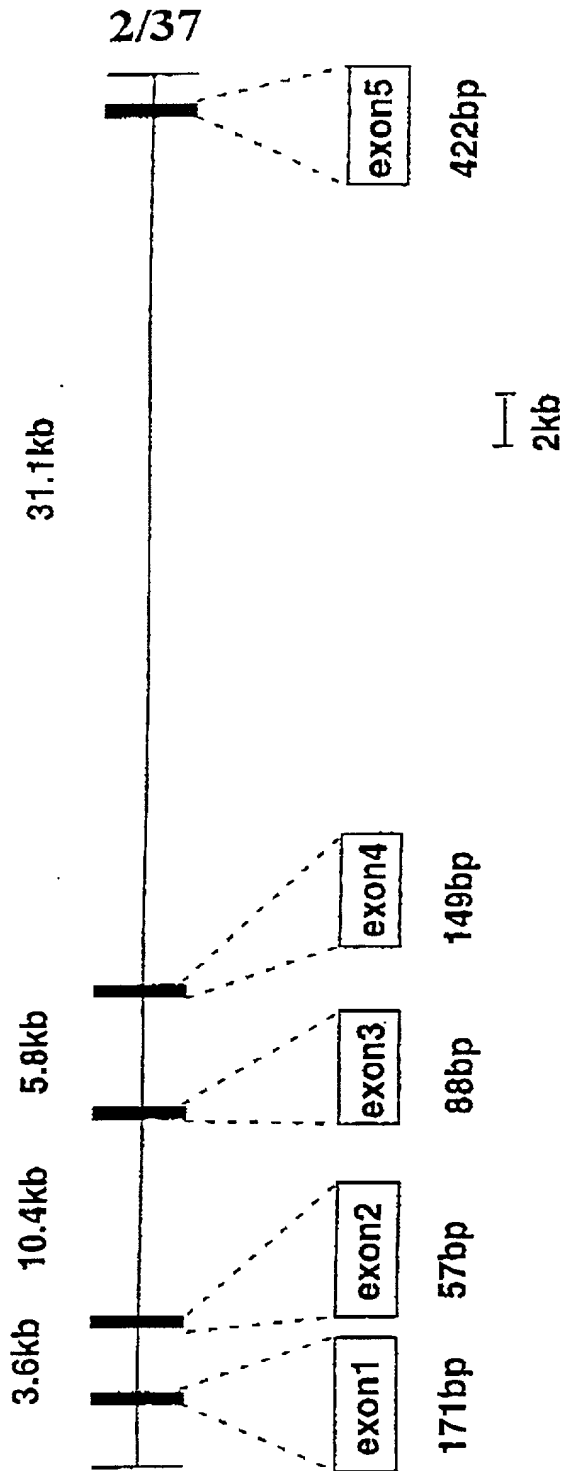


FIGURE 2

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Human Csl M S K Q P V S N V R A I Q A N I N I P N G A F R P G A G Q P P
 Mouse Csl M S K Q P I S N V R A I Q A N I N I P M G A F R P G A G Q P P
 Xenopus Csl M S K Q P A S N I R S I Q A N I N I P M G A F R P G A G Q P P

Human Csl R R K E C T P E V E E G V P P T - S D - - - E E K K P I P G
 Mouse Csl R R K E S T P E T E E G A P T T - S - - - E E K K P I P G
 Xenopus Csl K R K E F S T E E - E Q H V P T P E S E E K S E E K K P I P G

Human Csl A K K L P G P A V N L S E I Q N I K S E L K Y V P K A E Q .
 Mouse Csl M K K F P G P V V N L S E I Q N V K S E L K F V P K G E Q .
 Xenopus Csl A V K L P G P A F N L S E T .

FIGURE 3

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L.intestine
Uterus
Bladder
Sk.muscle
S.intestine
Kidney
Pancreas
Testes
Ovary
Spleen
Stomach
Liver
Lung
Heart
Thymus
S.gland
Brain
Tongue
Skull

Csl

Cyclo

FIGURE 4

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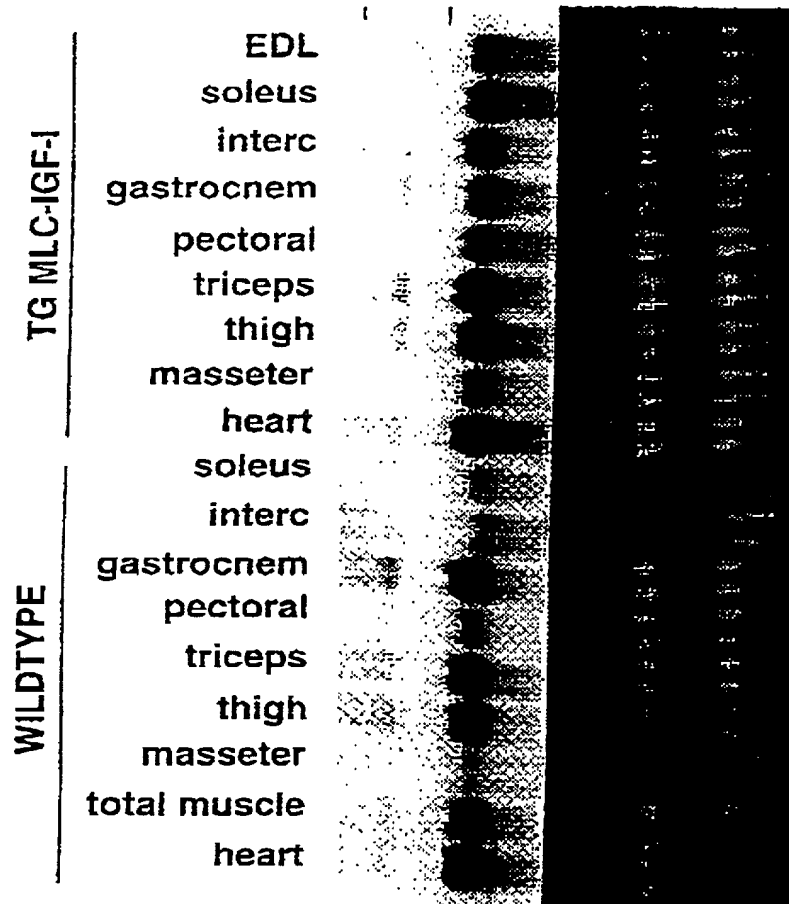


FIGURE 5

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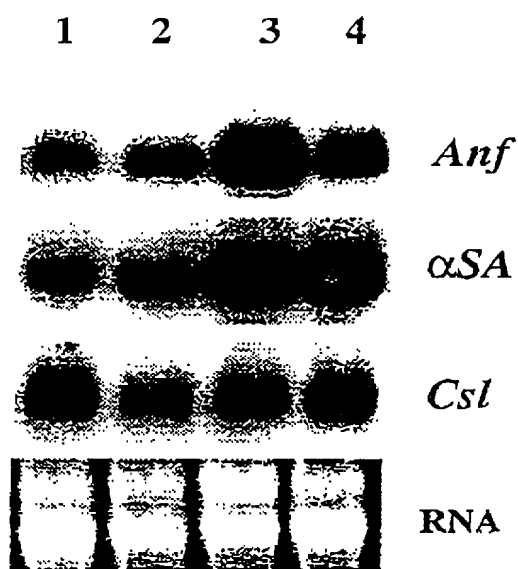


FIGURE 6

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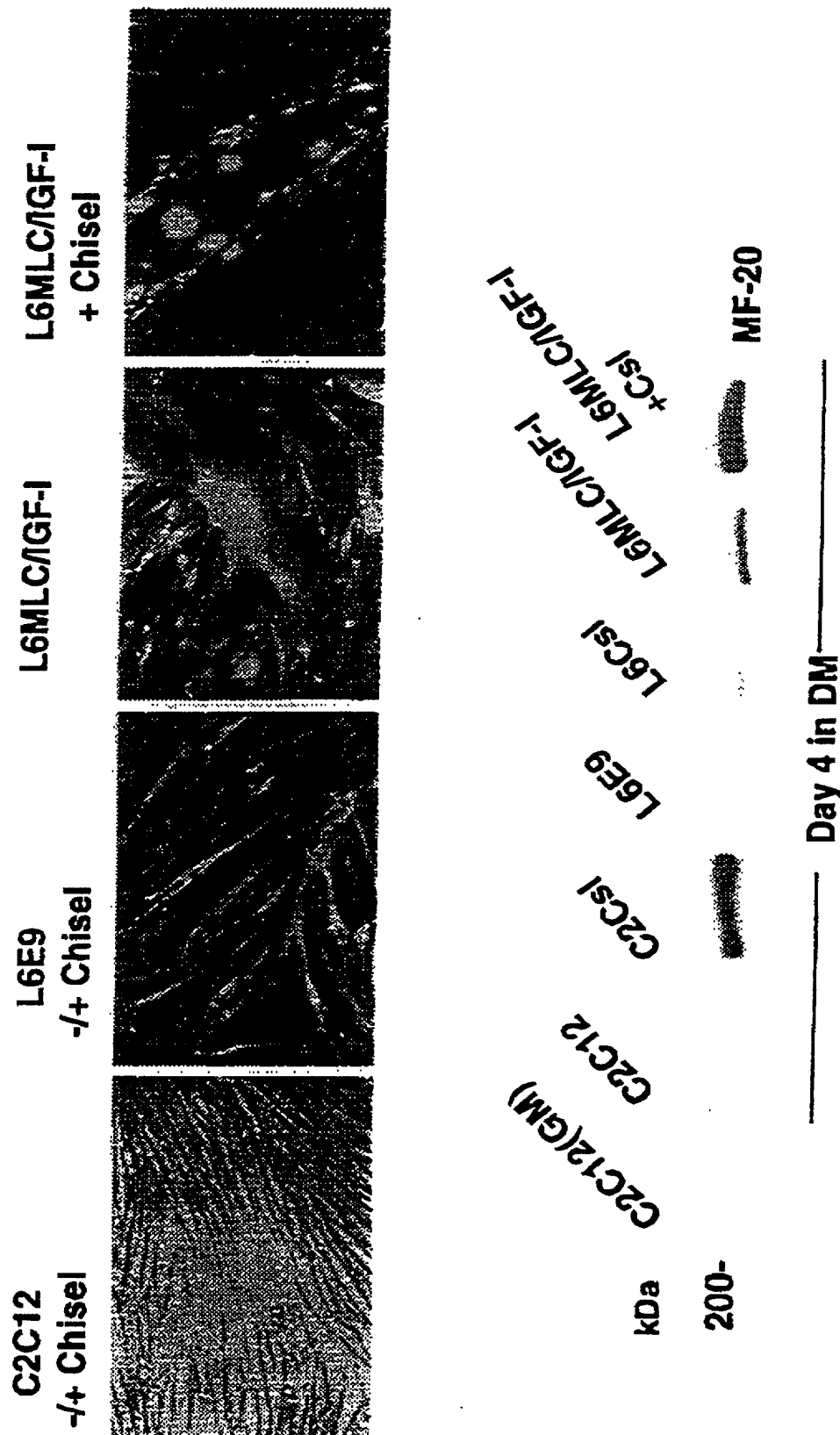


Figure 7

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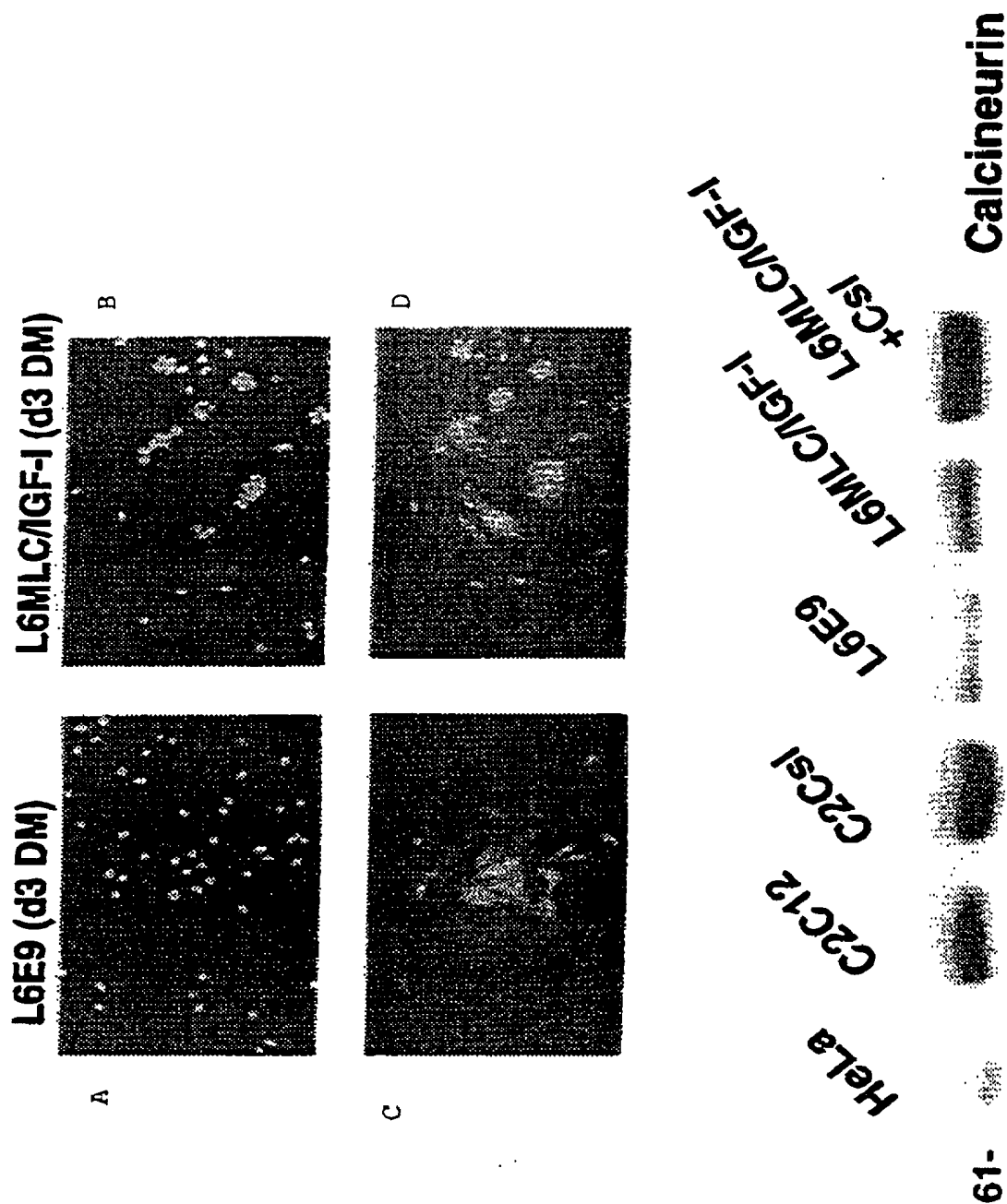


Figure 8

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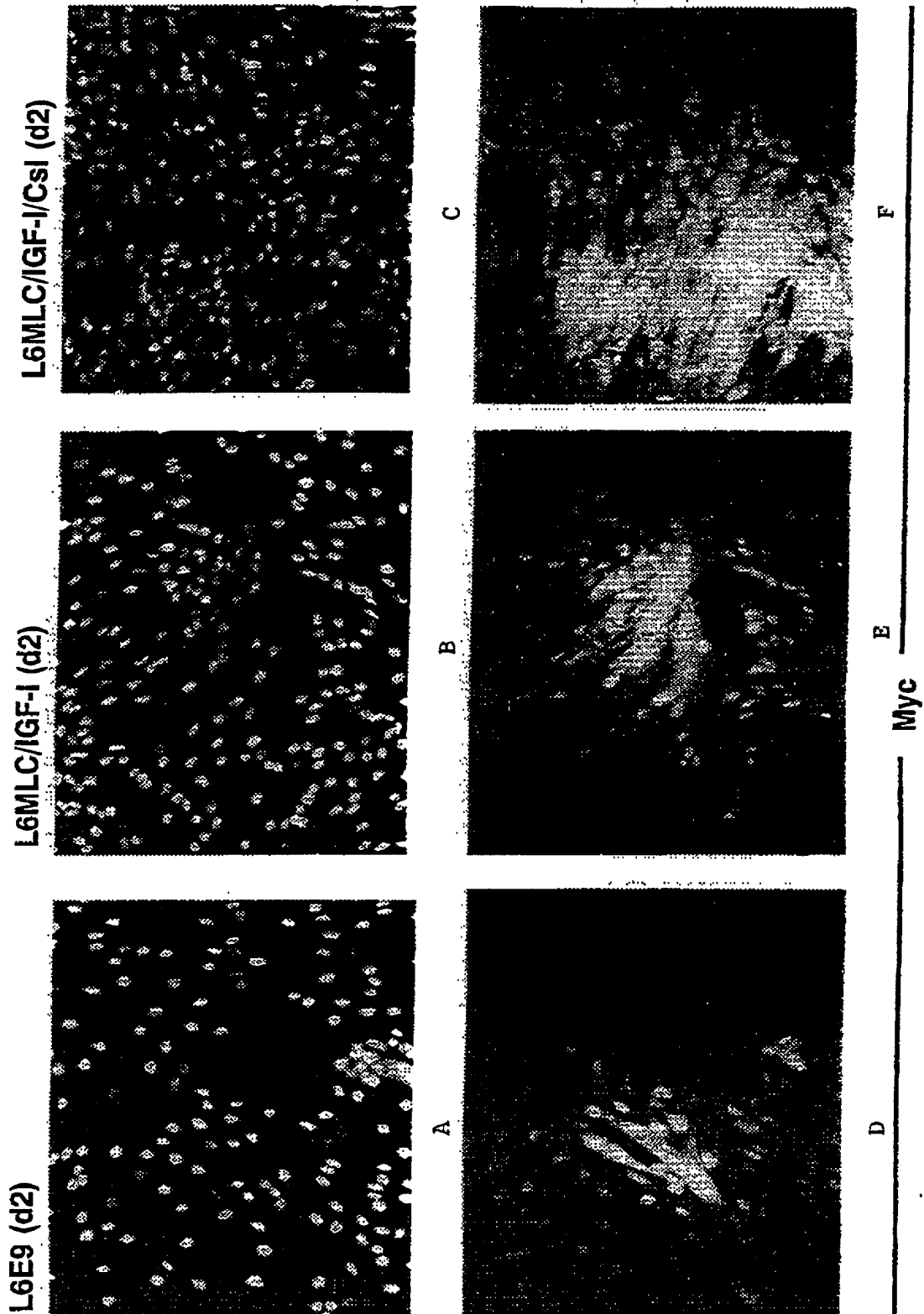


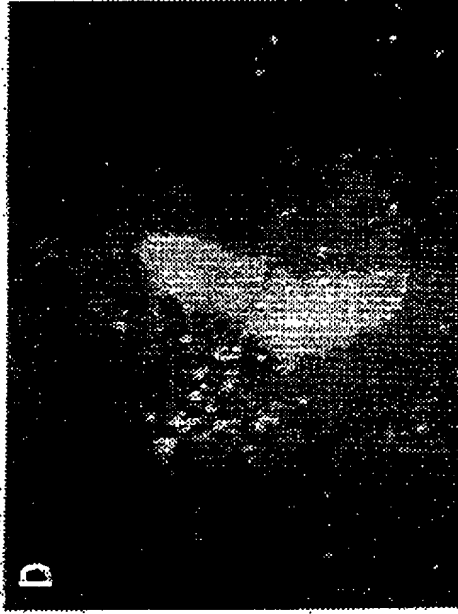
Figure 9

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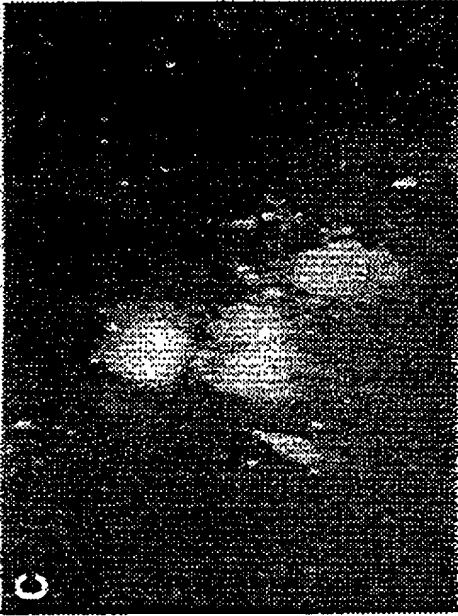
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L6MLC/IGF-I/Csl (d5)



L6MLC/IGF-I (d5)



Myc

Figure 10

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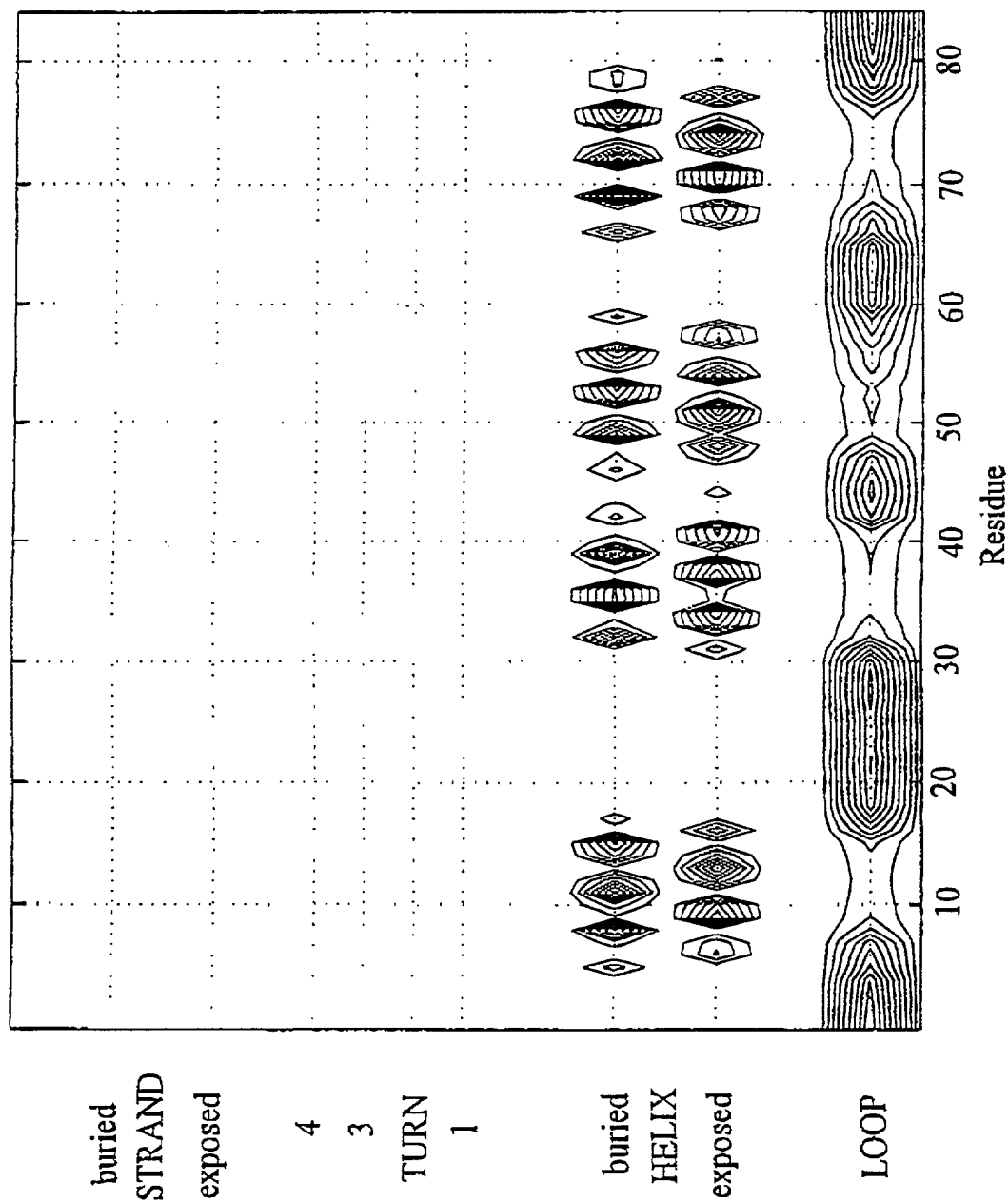


FIGURE 11

BMERC Protein Sequence Analysis System
<http://bmerc-www.bu.edu/psa/about.htm>

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Incomplete IQ Site

Binding to C-terminal lobe Binding to N-terminal lobe

N → C C ← N

Scallop MHC (R-MLC site): SVIQRNIRKwVLRlnwQwWkLYskVKpLLsIARQEEEmKE
 rat cardiac MHC (R-MLC site): LVIQWNIRAFMGvKNwPwMKLYfKIKpLLKSAETeKEMAN
 rat skeletal MHC (R-MLC site): FCIQYNIRAFMNvKHwPwMKLFFKIKpLLKSAETeKEMAT
 CALCINEURIN A (CnB site): ENNVMNIRQfNCSPHPYwLPnFMDVFTwSLPFVGEKRVTE

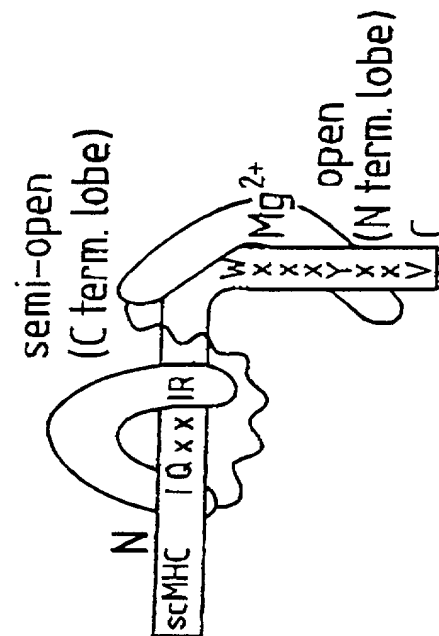
CONSENSUS:

IQxxIR WxxxYxxV
 FxxI

CALCINEURIN A mutations
 with reduced CnB binding:

A A

FIGURE 12



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Non-IQ Site

Binding to N-terminal lobe
C ← N

Binding to C-terminal lobe
C ← N

CaM Binding Sites

CALCINEURIN A: SEEDGFDGATAAARKEVIRWKIRAI GKMARVFSVLREESVS
SK MLCK (M13): KRRWKKNFIAVSAANRFKKISSGAL
Ca Pump (C24W): QILWERGLNRIQTQIRVVNAFRSS
CALSPERMIN: ARRLKAAVKAVVASSRLGS
MELLITIN: GIGAVLKVLTTGLPALISWIKRKRQQ

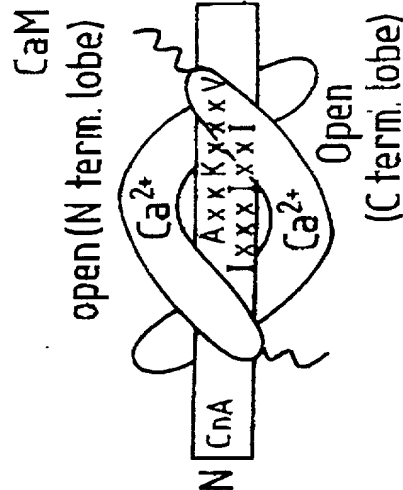


FIGURE 12

Alignments and binding modes taken from Houdusse and Cohen (1995) PNAS 92, 10644-10647 and Ikura et al. (1992) Science 256,632-638.
Calcineurin mutations found in Milan et al. (1994) Cell 79, 437-447.

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PAGE Analysis of Thrombin-Cleaved Bacterial GST-Chisel

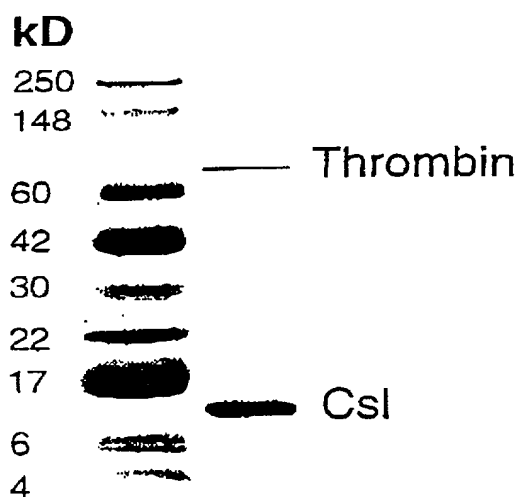


FIGURE 13

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Western Blot Analysis of COS Cell-Expressed and Bacterial Chisel with Native Chisel Antibody

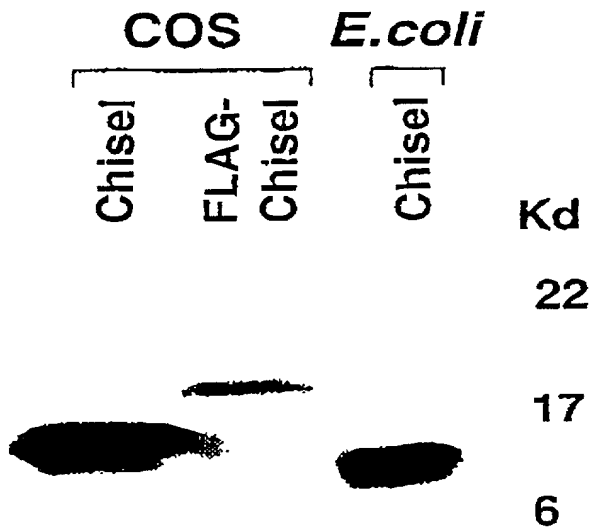


FIGURE 14

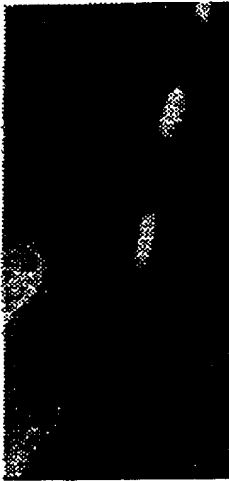
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G



H



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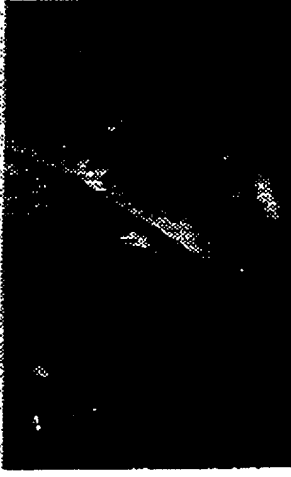
FLAG CHISEL



C



D



E



F

HOECHST

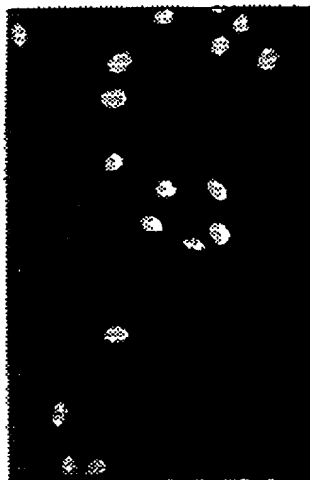


Figure 15

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FOOTNOTES

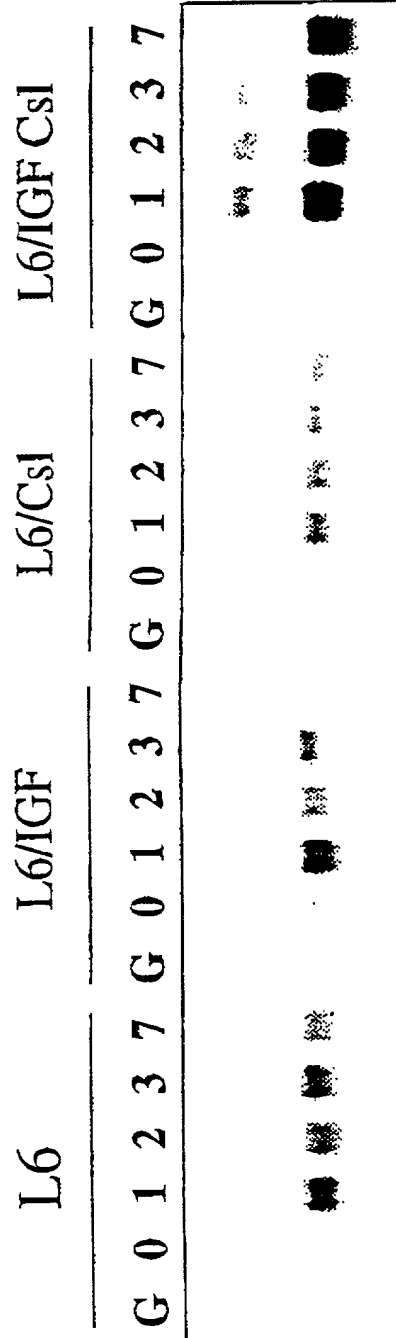


FIGURE 16

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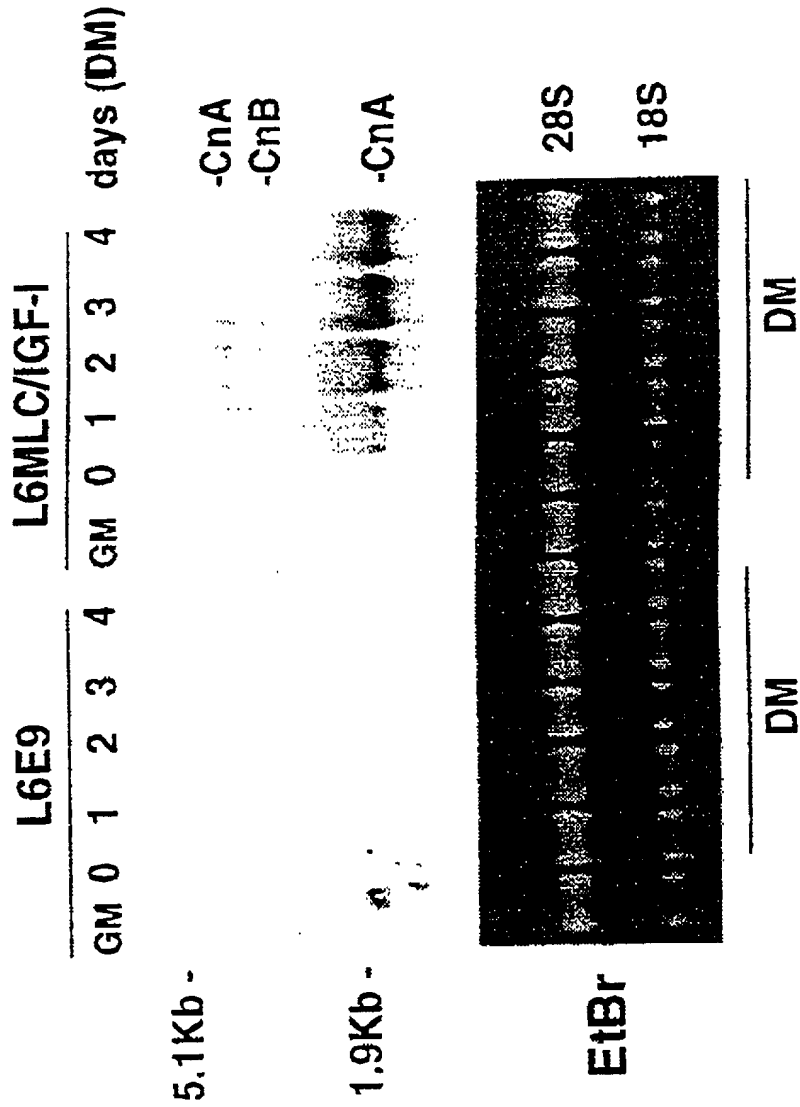


Figure 17 a

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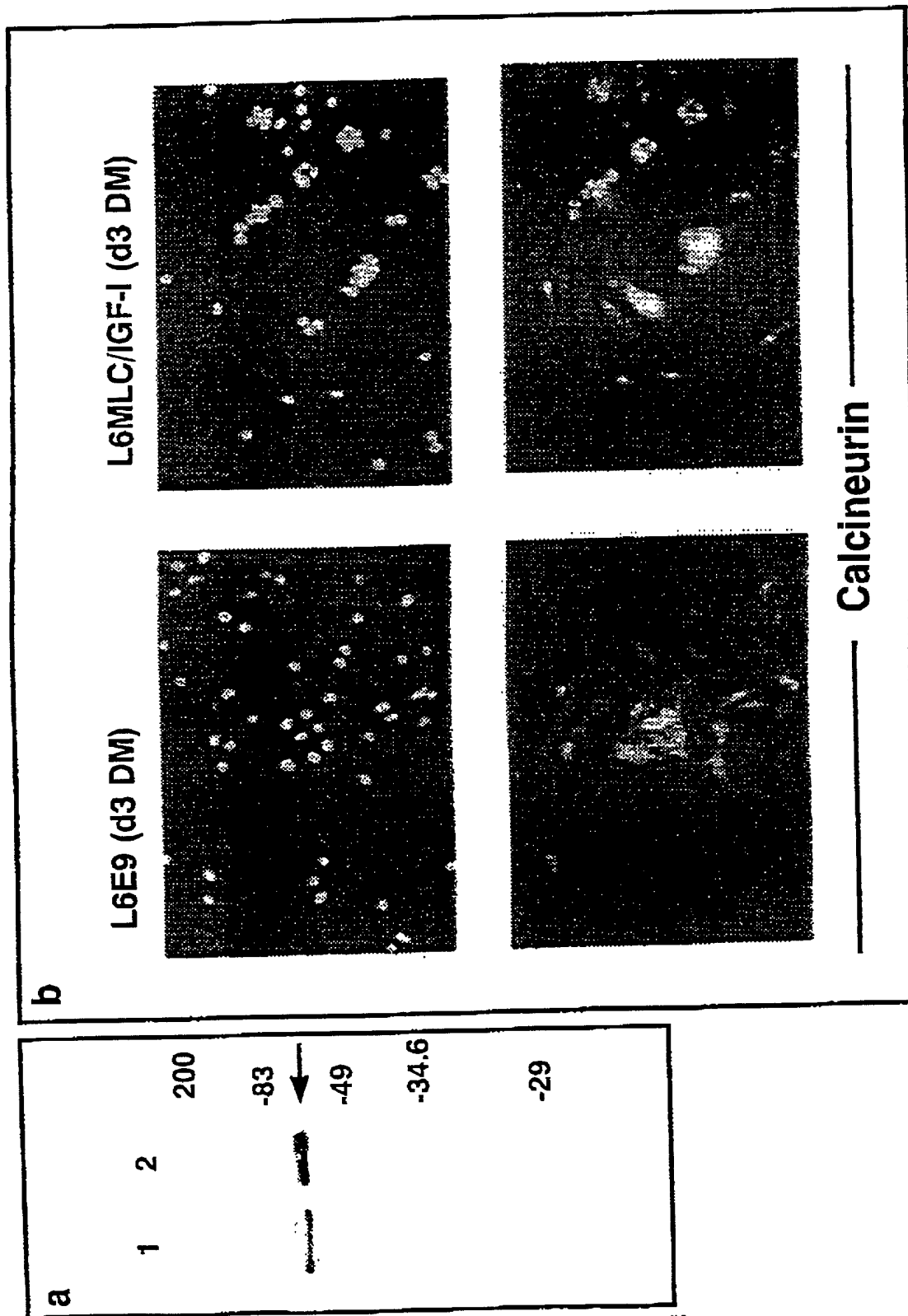


Figure 17b

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200 X

400 X

L6Myog/CnA (d2 DM)



L6E9 (d2 DM)

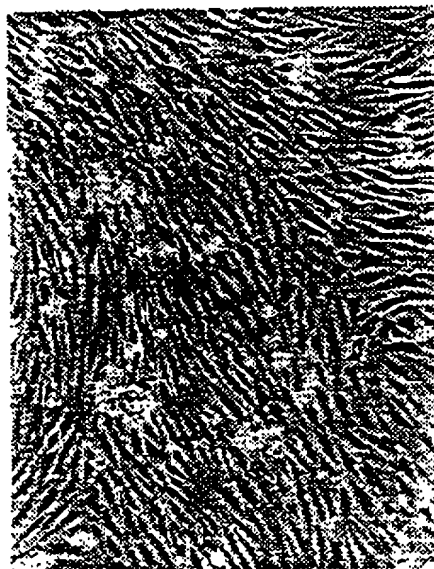
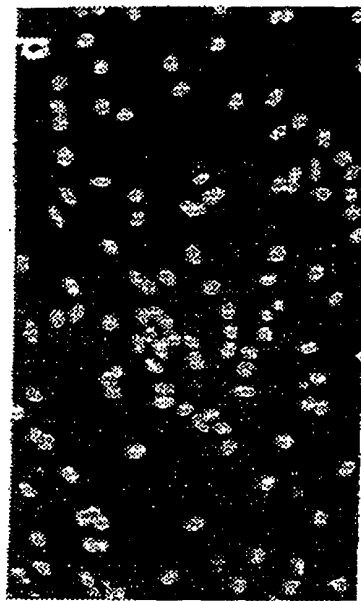


Figure 18a

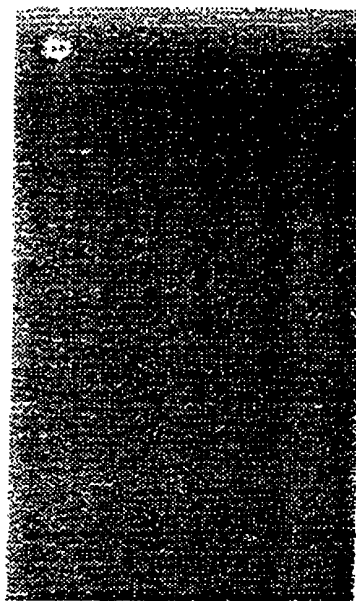
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Hoechst



HA



MF-20

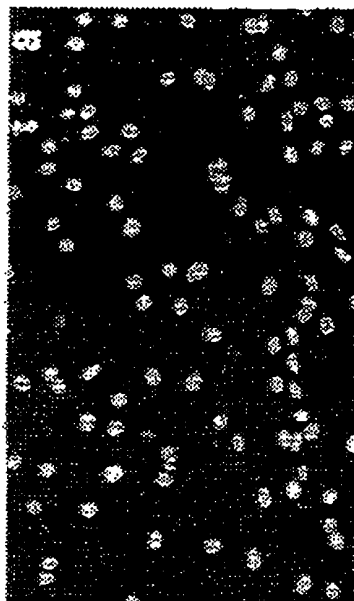


Figure 18b

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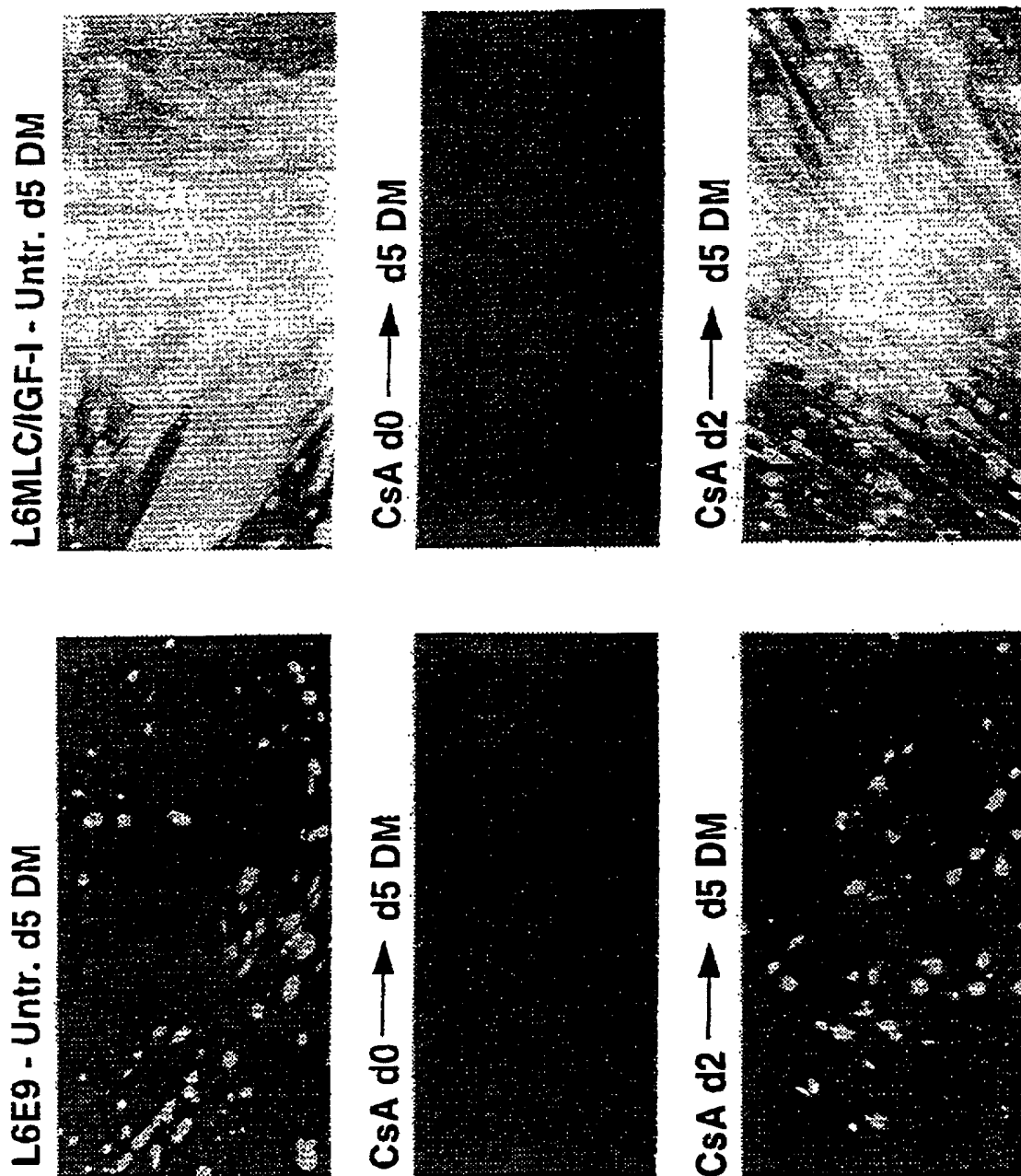


Figure 18c

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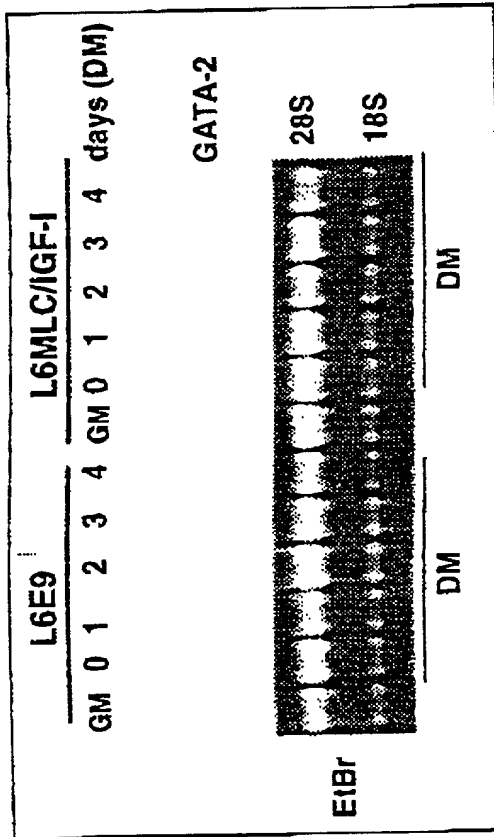


Figure 19a

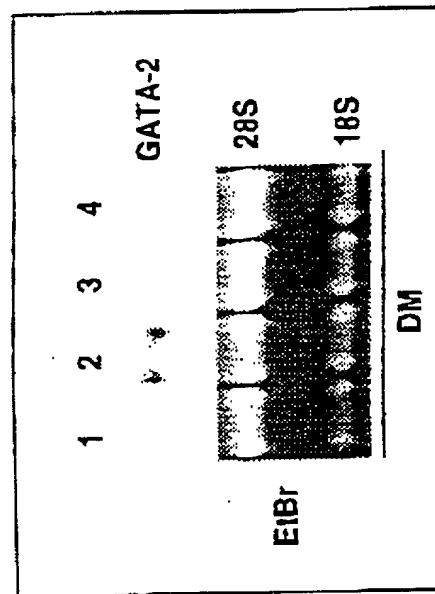


Figure 19b

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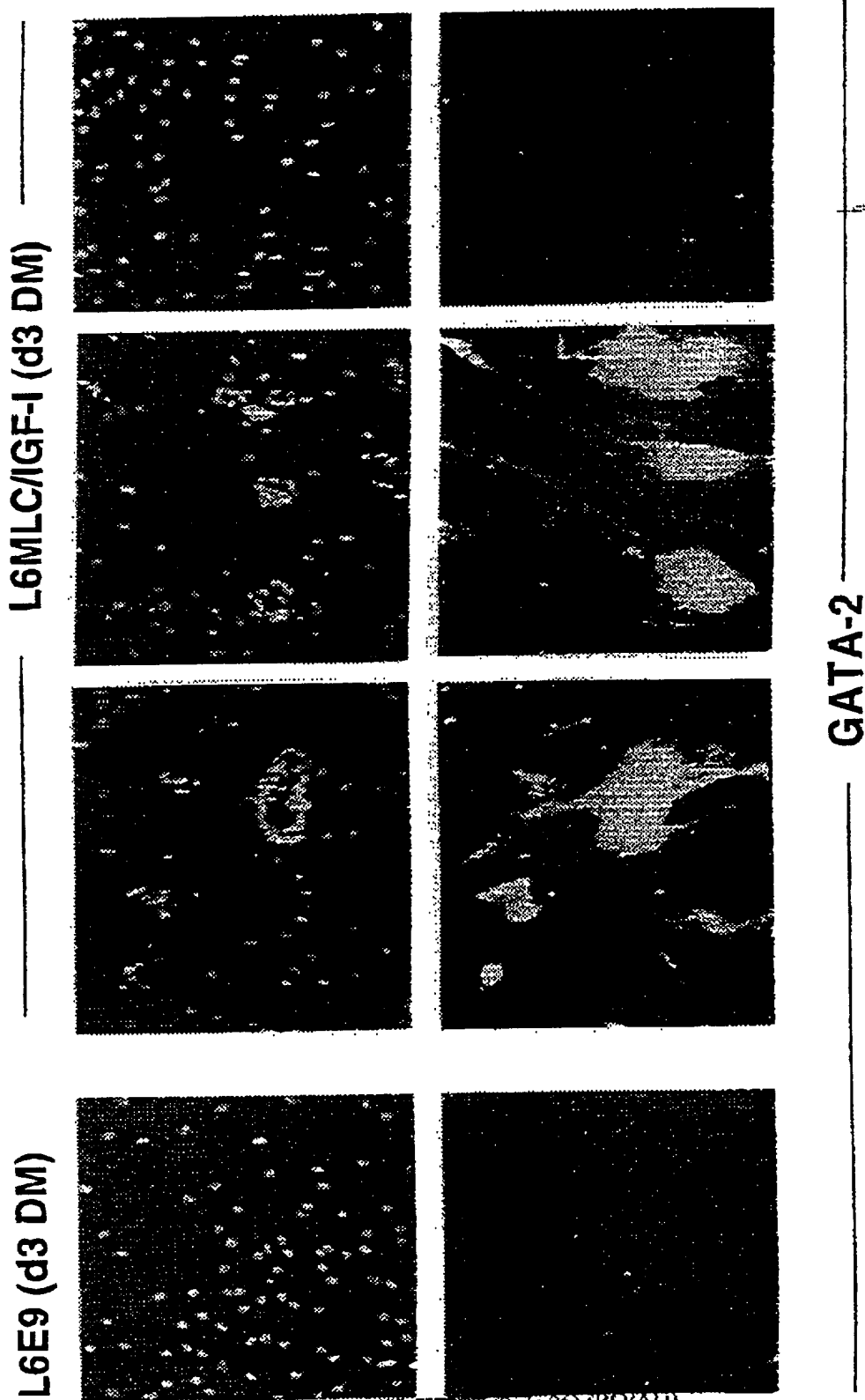


Figure 19c

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FIG. 19d

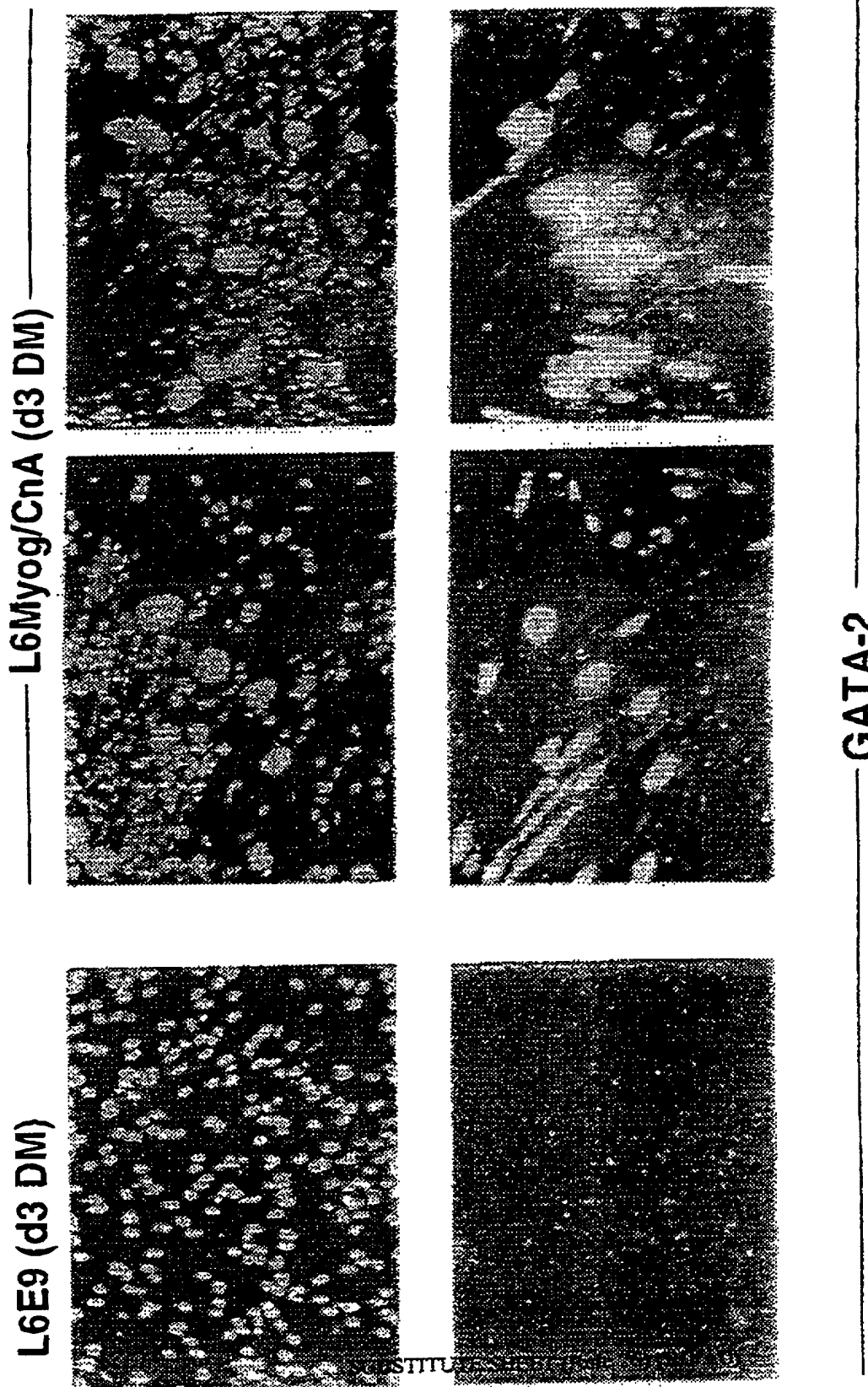


Figure 19d

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Control



IGF-1



IGF-1+ CsA

Figure 20 a

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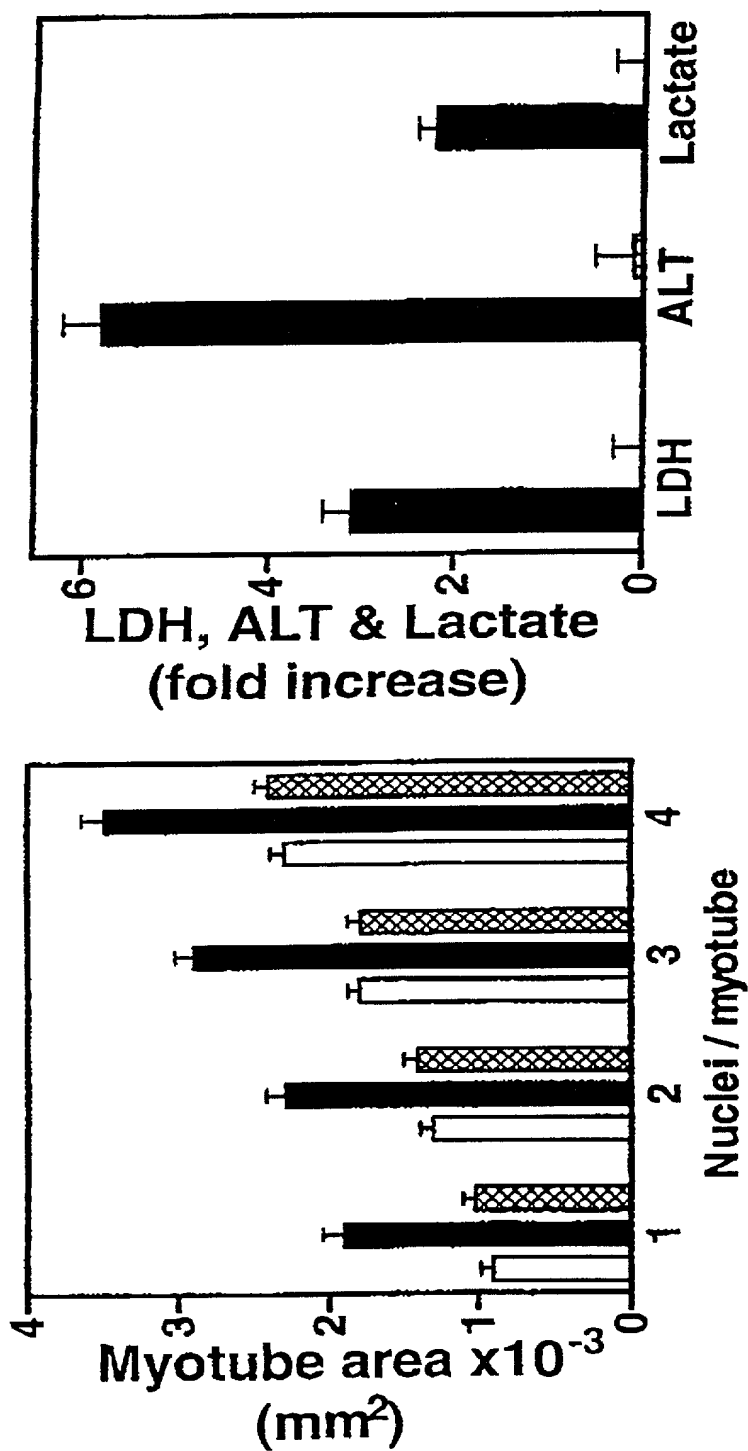


FIGURE 20B

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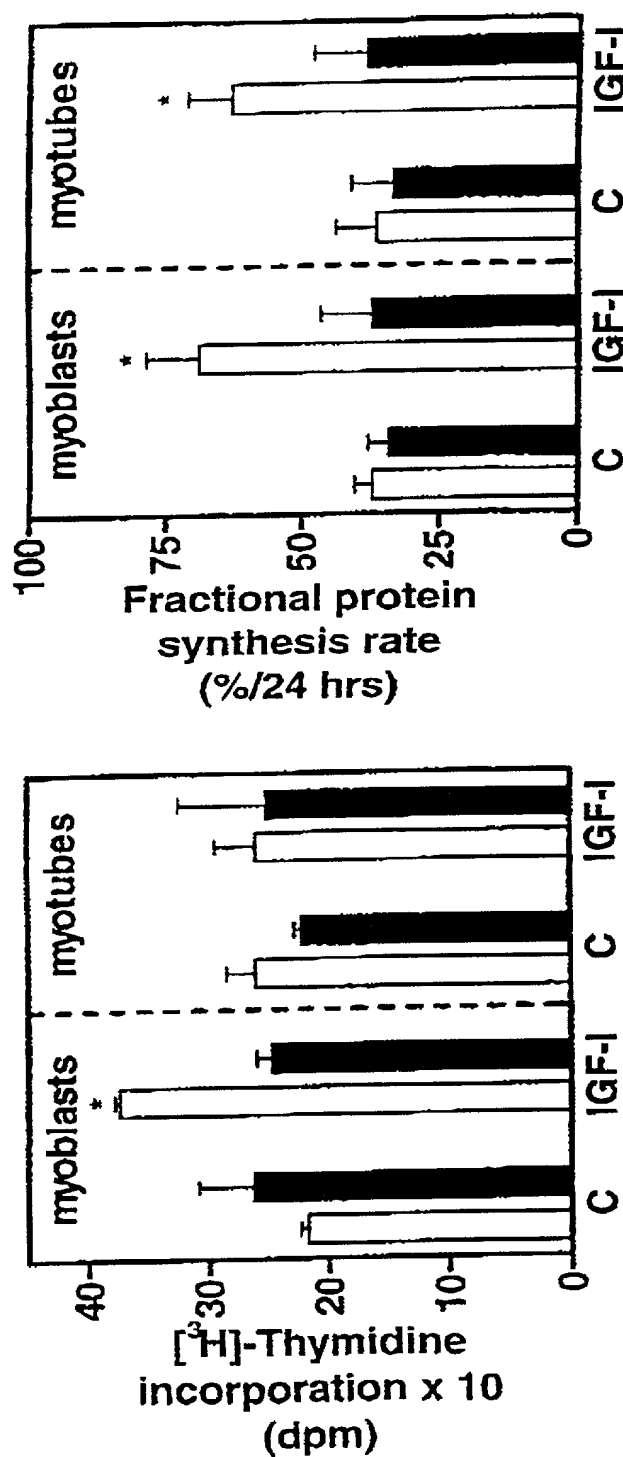


FIGURE 20C

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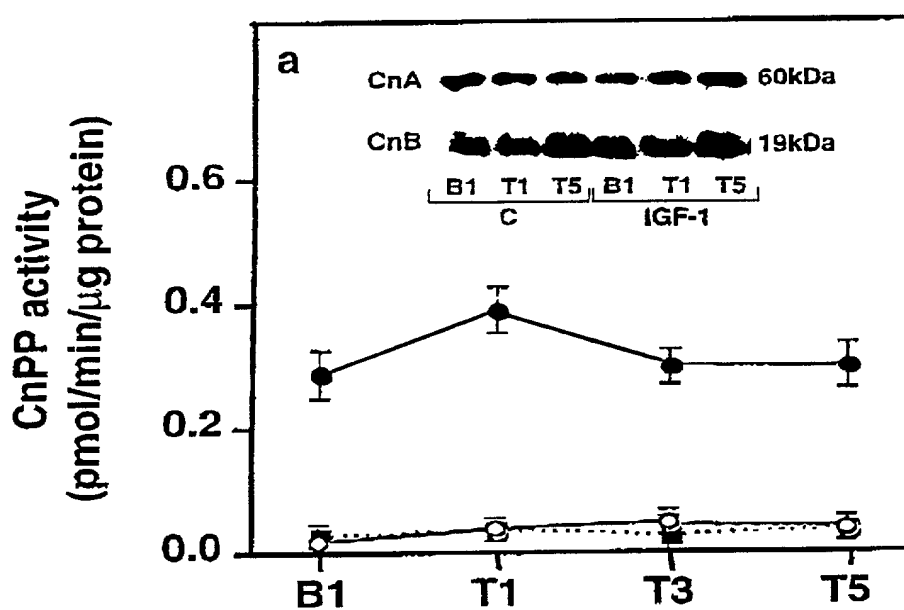


FIGURE 21

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PCT/AU99/00220

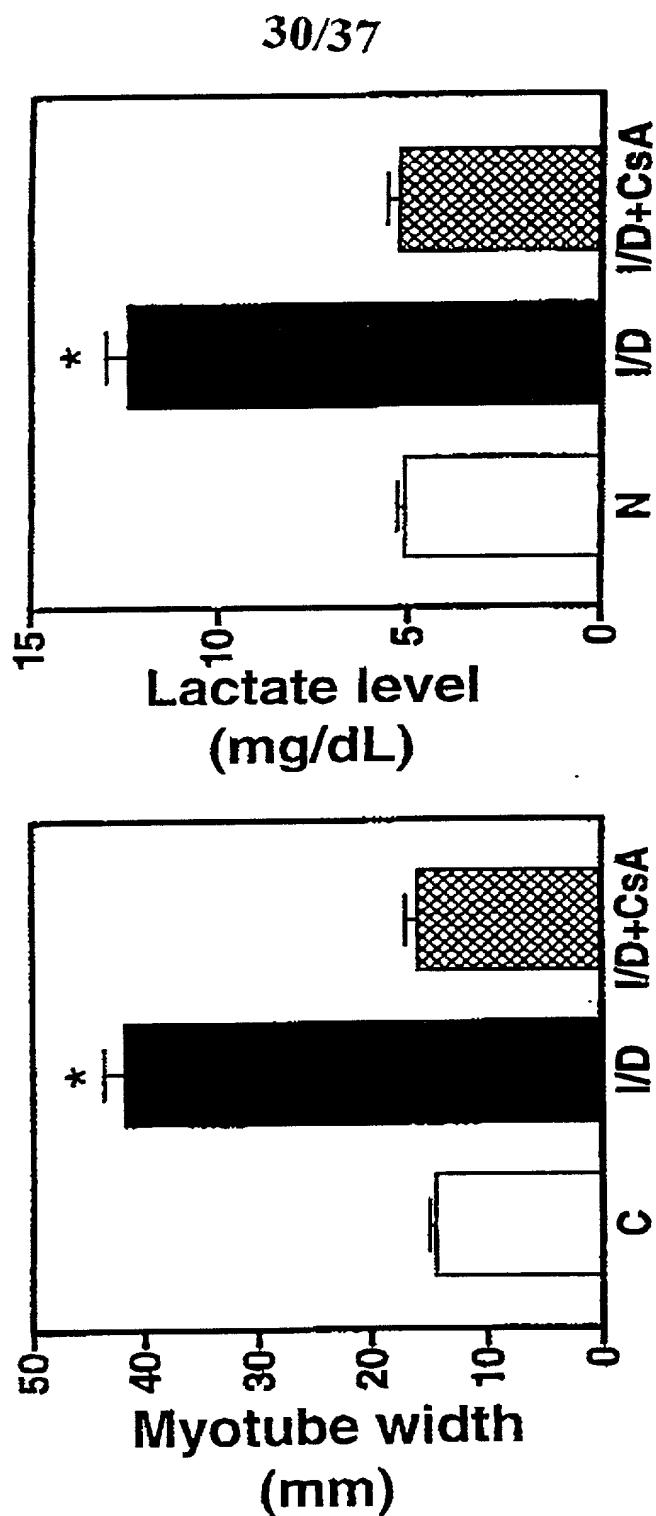


FIGURE 22A

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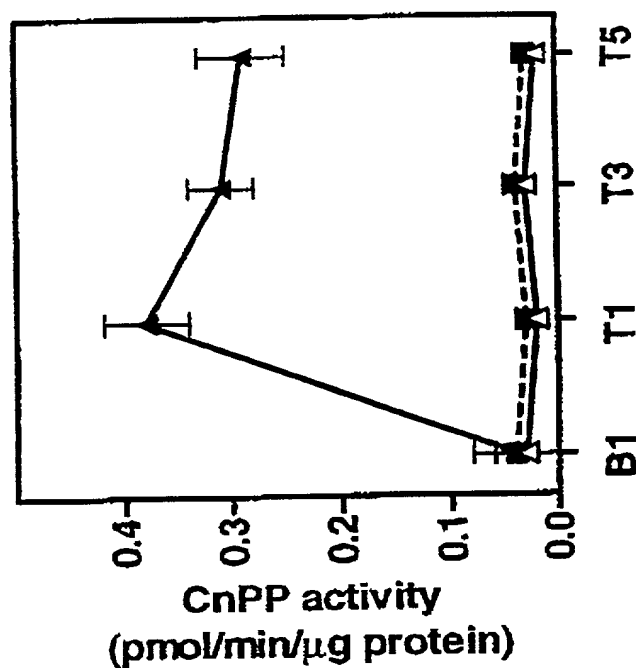
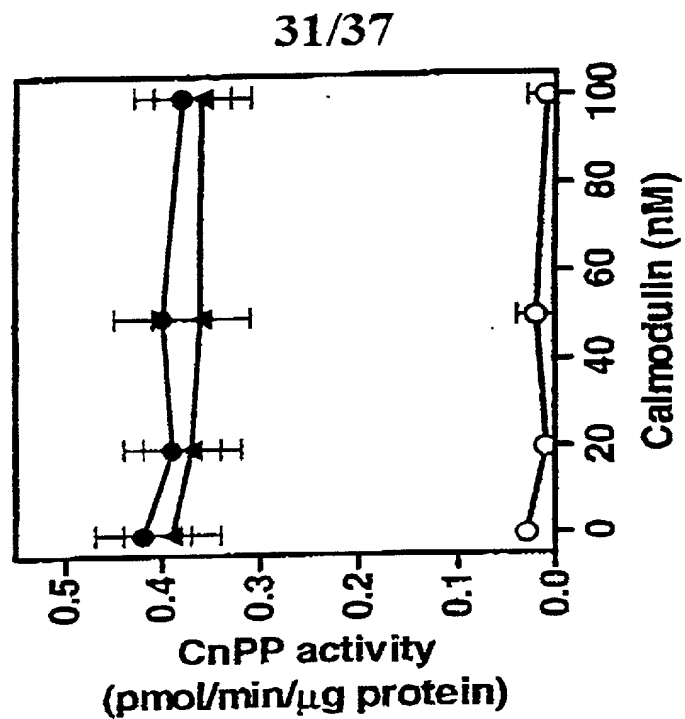


FIGURE 22B

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Figure 23 a

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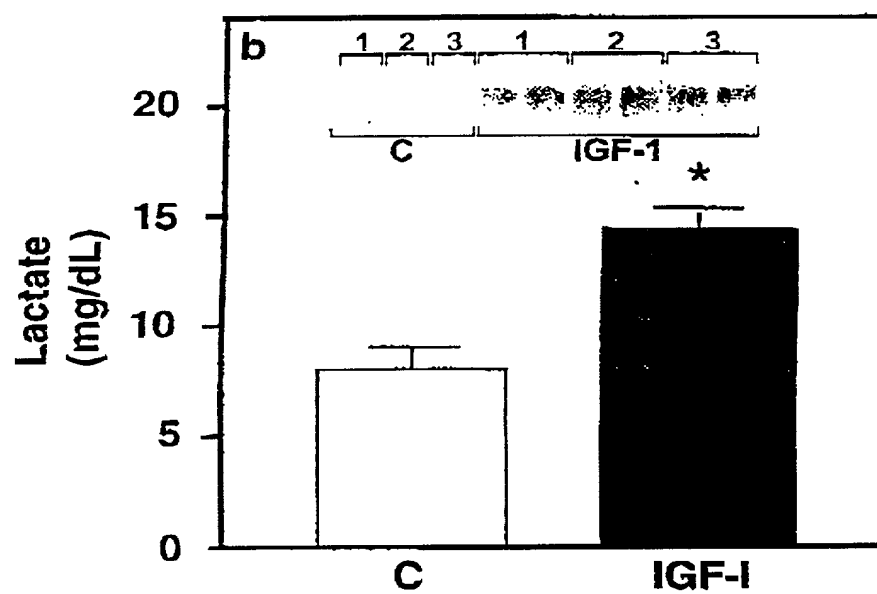


FIGURE 23B

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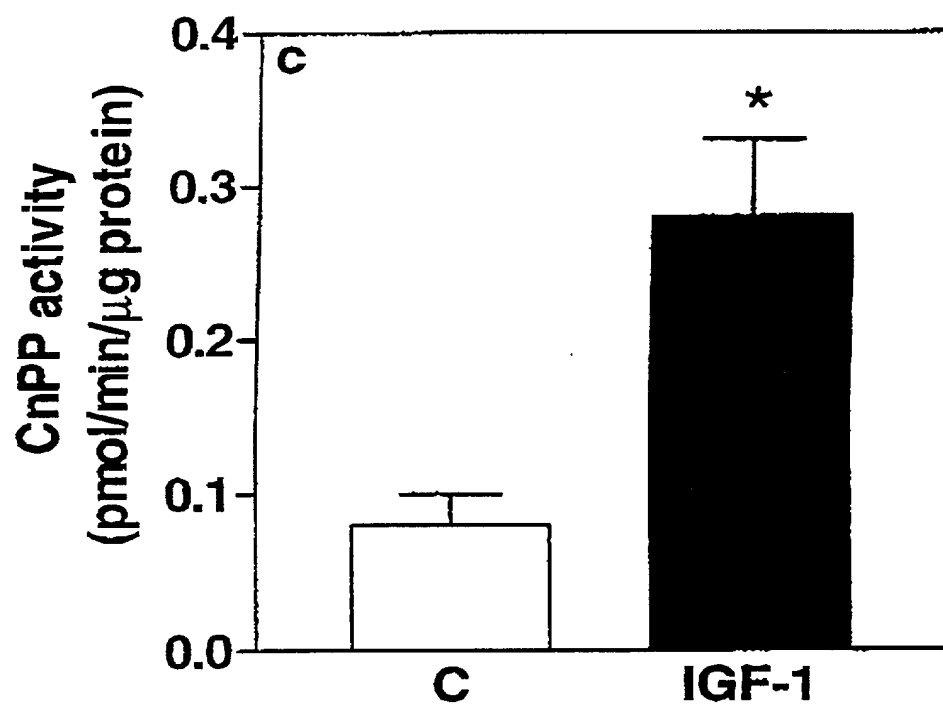
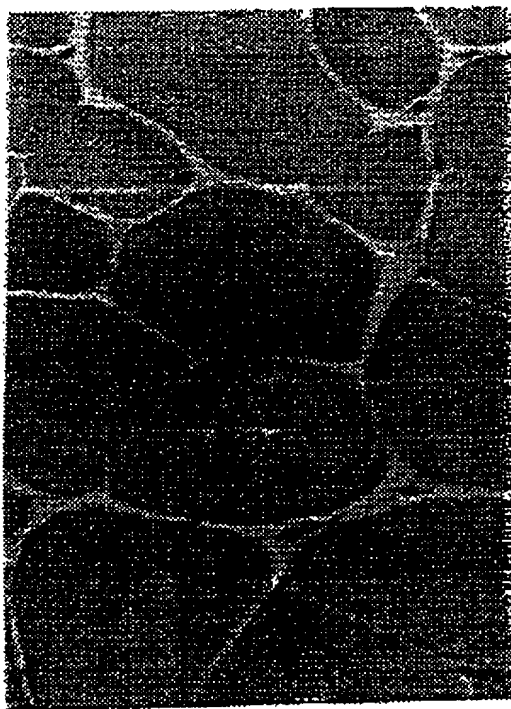


FIGURE 23C

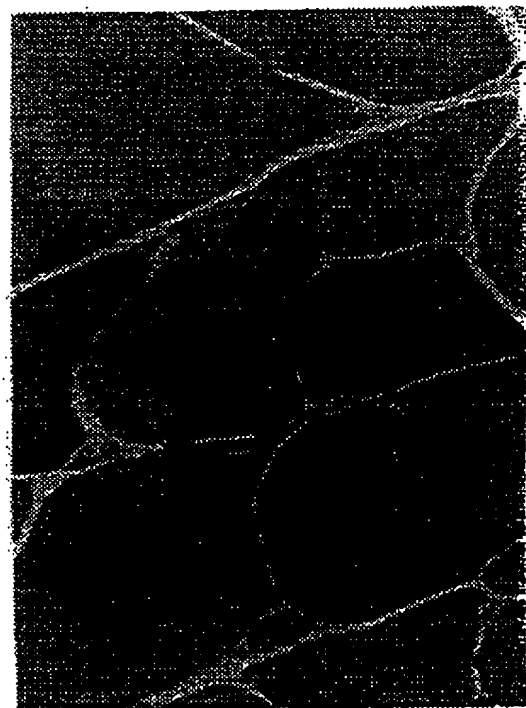
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Control



IGF-1

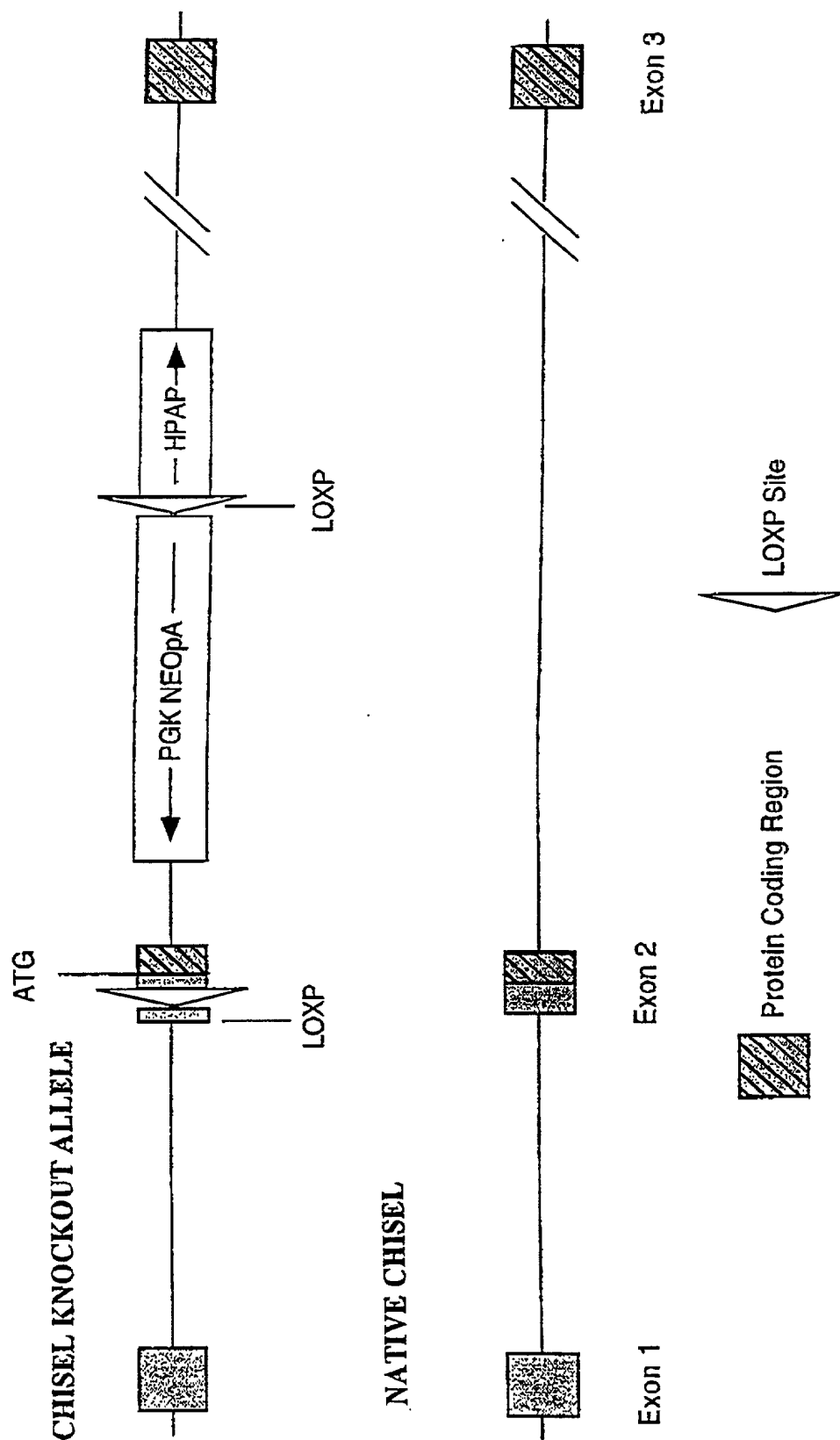
FIGURE 23D

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FIGURE 24



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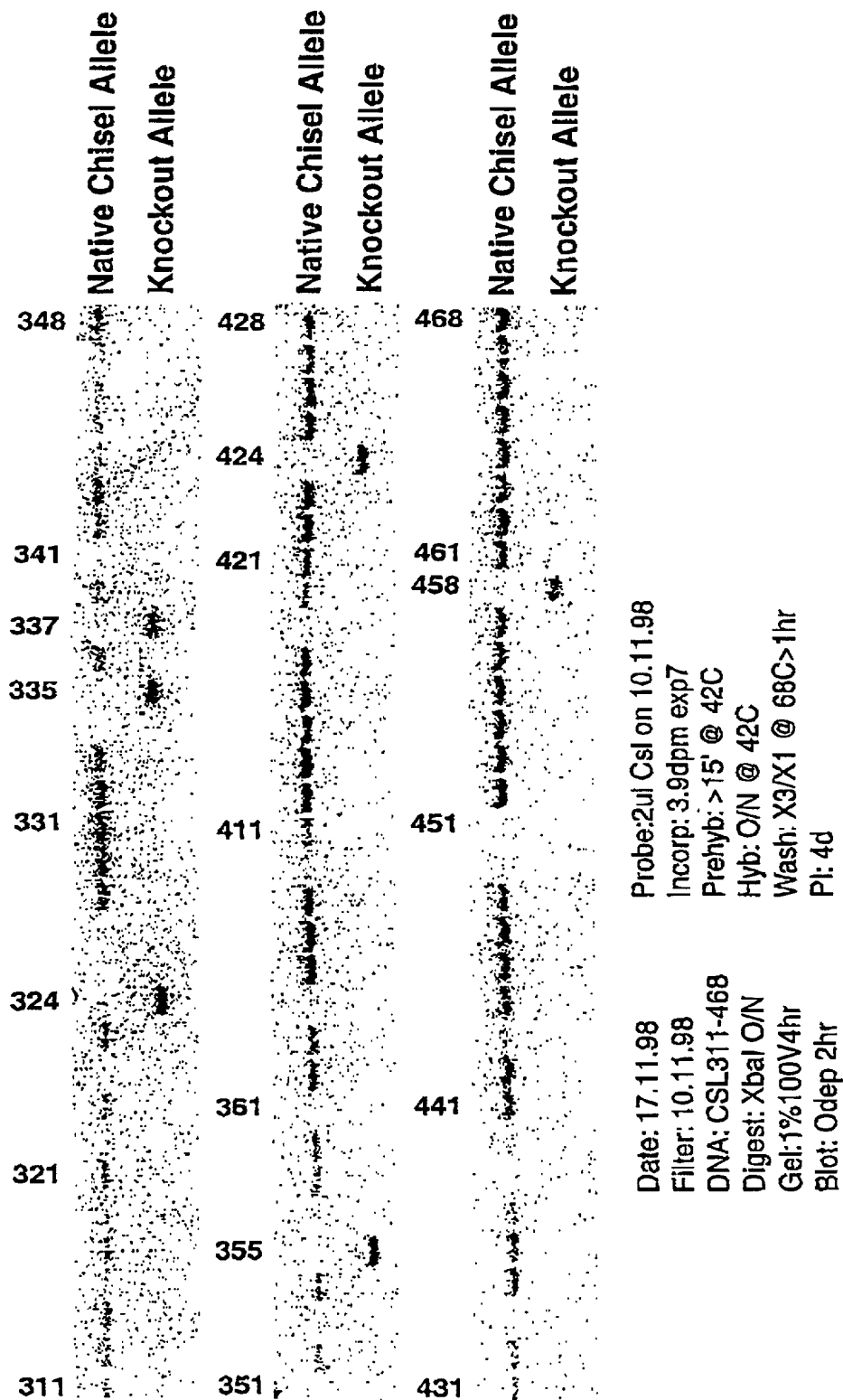


FIGURE 25

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled NOVEL MOLECULES EXPRESSED DURING MUSCLE DEVELOPMENT AND GENETIC SEQUENCES ENCODING THE SAME, the specification of which:

- ☐ is attached hereto.
☒ was filed on September 26, 2000 as Application Serial No. 09/647,019 and was amended on _____.
☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

U.S. Serial No.	Filing Date	Status
PCT/AU99/00220	March 26, 1999	Pending

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Application No.	Filing Date	Priority Claimed
Australia	PP 2634	March 27, 1998	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Yes <input type="checkbox"/> No

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Janis K. Fraser, Reg. No. 34,819
 J. Peter Fasse, Reg. No. 32,983
 Timothy A. French, Reg. No. 30,175
 Anita L. Meiklejohn, Reg. No. 35,283

Gary L. Creason, Reg. No. 34,310
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Address all telephone calls to JANIS K. FRASER at telephone number (617) 542-5070.

Address all correspondence to JANIS K. FRASER at:

TOTAL 572450

Combined Declaration and Power of Attorney

Page 2 of 2 Pages

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225 Franklin Street
Boston, MA 02110-2804

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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Full Name of Inventor: NADIA A. ROSENTHAL

Inventor's Signature: Nadia Rosenthal Date: Jan 19, 01
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Full Name of Inventor: STEPHEN J. PALMER

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 Australia

Attorney's Docket No.: 00786-407001

Combined Declaration and Power of Attorney

Page 2 of 3 Pages

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225 Franklin Street
Boston, MA 02110-2804

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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Date:

* 16-11-00

Full Name of Inventor: NADIA A. ROSENTHAL

Inventor's Signature:

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United States of America

Citizenship:

Post Office Address:

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United States of America

Date:

Full Name of Inventor: STEPHEN J. PALMER

Inventor's Signature: *

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Australia

Citizenship:

Post Office Address:

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Date:

* 16-11-00

16/11 '00 10:15

TX/RX NO. 0608

P04

Combined Declaration and Power of Attorney

Page 3 of 3 Pages

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Inventor's Signature: Antonio Musaro

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ITALY
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00135 ROMA
ITALY

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00786-407001

09/647019
430 Rec'd PCT/PTO 26 SEP 2000

WO 99/50410

PCT/AU99/00220

- 1 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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GENERAL HOSPITAL CORPORATION and
THE WALTER AND ELIZA HALL
INSTITUTE OF MEDICAL RESEARCH.

(ii) TITLE OF INVENTION:

NOVEL THERAPEUTIC MOLECULES
AND USES THEREFOR

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: DAVIES COLLISON CAVE
(B) STREET: 1 LITTLE COLLINS STREET
(C) CITY: MELBOURNE
(D) STATE: VICTORIA
(E) COUNTRY: AUSTRALIA
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: INTERNATIONAL APPLICATION
(B) FILING DATE: 26-MAR-1999
(C) CLASSIFICATION:

(vii) PREVIOUS APPLICATION DATA:

(A) APPLICATION NUMBER: PP2634/98
(B) FILING DATE: 27-MAR-1998

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: HUGHES, DR E JOHN L
(C) REFERENCE/DOCKET NUMBER: EJH/TDO/DK

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(C) TELEX: AA 31787

09/647019 043604

PCT/AU99/00220

(2) INFORMATION FOR SEQ ID NO:1:

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(ix)  FEATURE:
      (A) NAME/KEY: CDS
      (B) LOCATION: 199..453
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

[illegible]

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PCT/AU99/00220

- 3 -

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 85 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Ser Lys Gln Pro Ile Ser Asn Val Arg Ala Ile Gln Ala Asn Ile
 1           5           10           15
Asn Ile Pro Met Gly Ala Phe Arg Pro Gly Ala Gly Gln Pro Pro Arg
          20           25           30
Arg Lys Glu Ser Thr Pro Glu Thr Glu Glu Gly Ala Pro Thr Thr Ser
          35           40           45
Glu Glu Lys Lys Pro Ile Pro Gly Met Lys Lys Phe Pro Gly Pro Val
          50           55           60
Val Asn Leu Ser Glu Ile Gln Asn Val Lys Ser Glu Leu Lys Phe Val
          65           70           75           80
Pro Lys Gly Glu Gln
                   85

```

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 887 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 185..448

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

GGTTCTCAAT ACCGGGAGAG GCACAGAGCT ATTCAGCCA CATGAAAAGC ATCGGAATTG      60
AGATCGCAGC TCAGAGGACA CCGGGCGCCC CTCCACCTT CCAAGGAGCT TTGTATTCTT      120
GCATCTGGCT GCCTGGGACT TCCCTTAGGC AGTAAACAAA TACATAAAGC AGGGATAAGA      180
CTGC ATG AAT ATG TCG AAA CAG CCA GTT TCC AAT GTT AGA GCC ATC CAG      229
    Met Asn Met Ser Lys Gln Pro Val Ser Asn Val Arg Ala Ile Gln
      1           5           10           15
GCA AAT ATC AAT ATT CCA ATG GGA GCC TTT CGG CCA GGA GCA GGT CAA      277
Ala Asn Ile Asn Ile Pro Met Gly Ala Phe Arg Pro Gly Ala Gly Gln
          20           25           30
CCC CCC AGA AGA AAA GAA TGT ACT CCT GAA GTG GAG GAG GGT GTT CCT      325
Pro Pro Arg Arg Lys Glu Cys Thr Pro Glu Val Glu Glu Gly Val Pro
          35           40           45

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05647019.012601

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- 4 -

```

CCC ACC TCG GAT GAG GAG AAG AAG CCA ATT CCA GGA GCG AAG AAA CTT      373
Pro Thr Ser Asp Glu Glu Lys Lys Pro Ile Pro Gly Ala Lys Lys Leu
      50                      55                      60

CCA GGA CCT GCA GTC AAT CTA TCG GAA ATC CAG AAT ATT AAA AGT GAA      421
Pro Gly Pro Ala Val Asn Leu Ser Glu Ile Gln Asn Ile Lys Ser Glu
      65                      70                      75

CTA AAA TAT GTC CCC AAA GCT GAA CAG TAGTAGGAAG AAAAAAGGAT      468
Leu Lys Tyr Val Pro Lys Ala Glu Gln
      80                      85

TGATGTGAAG AAATAAGAG GCAGAAGATG GATTCAATAG CTCACTAAAA TTTTATATAT      528

TTGTATCATG ATTGTGAACC TCCTGAATGC CTGAGACTCT AGCAGAAATG GCCTGTTTGT      588

ACATTTTATAT CTCTTCCTTC TAGTTGGCTG TATTTCTTAC TTTATCTTCA TTTTGGCAC      648

CTCACAGAAC AAATTAGCCC ATAAATTCAA CACCTGGAGG GTGTGGTTTT GAGGAGGGAT      708

ATGATTTTAT GGAGAATGAT ATGGCAATGT GCCTAACGAT TTTGATGAAA AGTTTCCCAA      768

GCTACTTCCT ACAGTATTTT GGTCAATATT TGAATGCGT TTTAGTTCTT CACCTTTTAA      828

ATTATGTCAC TAACTTTGT ATGAGTTCAA ATAAATATTT GACTAAATGT AAAATGTGA      887

```

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 88 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Asn Met Ser Lys Gln Pro Val Ser Asn Val Arg Ala Ile Gln Ala
  1                      5                      10                      15

Asn Ile Asn Ile Pro Met Gly Ala Phe Arg Pro Gly Ala Gly Gln Pro
      20                      25                      30

Pro Arg Arg Lys Glu Cys Thr Pro Glu Val Glu Glu Gly Val Pro Pro
      35                      40                      45

Thr Ser Asp Glu Glu Lys Lys Pro Ile Pro Gly Ala Lys Lys Leu Pro
      50                      55                      60

Gly Pro Ala Val Asn Leu Ser Glu Ile Gln Asn Ile Lys Ser Glu Leu
      65                      70                      75                      80

Lys Tyr Val Pro Lys Ala Glu Gln
      85

```

F09270"6T04960

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- 5 -

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 75 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met Ser Lys Gln Pro Ala Ser Asn Ile Arg Ser Ile Gln Ala Asn Ile
1           5           10           15
Asn Ile Pro Met Gly Ala Phe Arg Pro Gly Ala Gly Gln Pro Pro Lys
20           25           30
Arg Lys Glu Phe Ser Thr Glu Glu Glu Gln His Val Pro Thr Pro Glu
35           40           45
Ser Glu Glu Lys Ser Glu Glu Lys Lys Pro Ile Pro Gly Ala Val Lys
50           55           60
Leu Pro Gly Pro Ala Phe Asn Leu Ser Glu Thr
65           70           75

```

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 887 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

GGTTCTCAAT ACCGGGAGAG GCACAGAGCT ATTTTCAGCCA CATGAAAAGC ATCGGAATTG      60
AGATCGCAGC TCAGAGGACA CCGGGCGCCC CTTCACCTT CCAAGGAGCT TTGTATTCTT      120
GCATCTGGCT GCCTGGGACT TCCCTTAGGC AGTAAACAAA TACATAAAGC AG      172

```

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 887 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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GGATAAGACT GCATGAATAT GTCGAAACAG CCAGTTTCCA ATGTTAGAGC CATCCAG      57

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 2009-09-01 15:13:26

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PCT/AU99/00220

- 6 -

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 887 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCAAATATCA ATATTCCAAT GGGAGCCTTT CGGCCAGGAG CAGGTCAACC CCCCAGAAGA 60
 AAAGAATGTA CTCCTGAAGT GGAGGAG 87

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 887 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGTGTTCCTC CCACCTCGGA TGAGGAGAAG AAGCCAATTC CAGGAGCGAA GAAACTTCCA 60
 GGACCTGCAG TCAATCTATC GGAAATCCAG AATATTAAAA GTGAACTAAA ATATGTCCCC 120
 AAAGCTGAAC AGTAGTAGGA AGAAAAAAG 149

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 887 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GATTGATGTG AAGAAATAAA GAGGCAGAAG ATGGATTCAA TAGCTCACTA AAATTTTATA 60
 TATTTGTATG ATGATTGTGA ACCTCCTGAA TGCCTGAGAC TCTAGCAGAA ATGGCCTGTT 120
 TGTACATTTA TATCTCTTCC TTCTAGTTGG CTGTATTTCT TACTTTATCT TCATTTTGGG 180
 CACCTCACAG AACAAATTAG CCCATAAATT CAACACCTGG AGGGTGTGGT TTTGAGGAGG 240
 GATATGATTT TATGGAGAAT GATATGGCAA TGTGCCTAAC GATTTTGATG AAAAGTTTCC 300
 CAAGCTACTT CCTACAGTAT TTTGGTCAAT ATTTGGAATG CGTTTGTAGTT CTTACCTTT 360
 TAAATTATGT CACTAAACTT TGTATGAGTT CAAATAAATA TTTGACTAAA TGTAATAATGT 420
 GA 422

T0927049.012004

SEQUENCE LISTING

<110> Harvey, Richard P.
Palmer, Stephen J.
Rosenthal, Nadia A.
Musaro, Antonio

<120> NOVEL MOLECULES EXPRESSED DURING MUSCLE
DEVELOPMENT AND GENETIC SEQUENCES ENCODING THE SAME

<130> 12525-407001

<140> 09/647,019

<141> 2000-09-26

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<151> 1999-03-26

<150> AU PP2634/98

<151> 1998-03-27

<160> 20

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<210> 1

<211> 778

<212> DNA

<213> Mus musculus

<220>

<221> CDS

<222> (199)...(453)

<400> 1

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gtgtttttgc acctggccgc ctgggactgt cctcaggcag taaaccaatc cagagagcag	180
ggctaagacc ttgtgaat atg tcg aag cag cca att tcc aac gtc aga gcc	231
Met Ser Lys Gln Pro Ile Ser Asn Val Arg Ala	
1 5 10	
atc cag gcg aat atc aat att cca atg gga gcc ttt cgt ccg gga gct	279
Ile Gln Ala Asn Ile Asn Ile Pro Met Gly Ala Phe Arg Pro Gly Ala	
15 20 25	
ggg cag cct ccc aga agg aaa gag agt act cct gaa act gag gag gga	327
Gly Gln Pro Pro Arg Arg Lys Glu Ser Thr Pro Glu Thr Glu Glu Gly	
30 35 40	
gct cct acc acc tca gag gaa aag aag cca att cct gga atg aag aaa	375
Ala Pro Thr Thr Ser Glu Glu Lys Lys Pro Ile Pro Gly Met Lys Lys	
45 50 55	
ttt cca gga cct gtt gtc aac ttg tct gag atc caa aat gtt aaa agt	423

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Phe Pro Gly Pro Val Val Asn Leu Ser Glu Ile Gln Asn Val Lys Ser
60 65 70 75

gaa ctg aaa ttt gtc ccc aaa ggt gaa cag tagtcgaaag gacacaaaag 473
Glu Leu Lys Phe Val Pro Lys Gly Glu Gln
80 85

ttcacattgg atgcttagaa tcaggagatg catttcgttg acgtgttttt ccaagggaga 533
aaaaacaatg ggttgaaata aacaacttcc tgaacatttt atacatttgt atgatgatca 593
caaacctcct gaatgcccaa gactctagca aaaatatcct gtttgtacat ttatatattct 653
tccttttact tggttgcatt tctcacttta gctacatttt tggcaccttg tagagcaaat 713
cagcacacga atttacaacc tgggaagtgt ggttttgagg agagatgtga tttttatgaa 773
ggggg 778

<210> 2
<211> 85
<212> PRT
<213> Mus musculus

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Asn Ile Pro Met Gly Ala Phe Arg Pro Gly Ala Gly Gln Pro Pro Arg
20 25 30
Arg Lys Glu Ser Thr Pro Glu Thr Glu Glu Gly Ala Pro Thr Thr Ser
35 40 45
Glu Glu Lys Lys Pro Ile Pro Gly Met Lys Lys Phe Pro Gly Pro Val
50 55 60
Val Asn Leu Ser Glu Ile Gln Asn Val Lys Ser Glu Leu Lys Phe Val
65 70 75 80
Pro Lys Gly Glu Gln
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<210> 3
<211> 887
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (185)...(448)

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gcattctggct gcctgggact tcccttaggc agtaaacaaa tacataaagc agggataaga 180
ctgc atg aat atg tcg aaa cag cca gtt tcc aat gtt aga gcc atc cag 229
Met Asn Met Ser Lys Gln Pro Val Ser Asn Val Arg Ala Ile Gln
1 5 10 15

gca aat atc aat att cca atg gga gcc ttt cgg cca gga gca ggt caa 277
Ala Asn Ile Asn Ile Pro Met Gly Ala Phe Arg Pro Gly Ala Gly Gln
20 25 30

ccc ccc aga aga aaa gaa tgt act cct gaa gtg gag gag ggt gtt cct 325
Pro Pro Arg Arg Lys Glu Cys Thr Pro Glu Val Glu Glu Gly Val Pro
35 40 45

ccc acc tcg gat gag gag aag aag cca att cca gga gcg aag aaa ctt 373
 Pro Thr Ser Asp Glu Glu Lys Lys Pro Ile Pro Gly Ala Lys Lys Leu
 50 55 60

cca gga cct gca gtc aat cta tcg gaa atc cag aat att aaa agt gaa 421
 Pro Gly Pro Ala Val Asn Leu Ser Glu Ile Gln Asn Ile Lys Ser Glu
 65 70 75

cta aaa tat gtc ccc aaa gct gaa cag tagtaggaag aaaaaaggat 468
 Leu Lys Tyr Val Pro Lys Ala Glu Gln
 80 85

tgatgtgaag aaataaagag gcagaagatg gattcaatag ctcactaaaa ttttatatat 528
 ttgtatgatg attgtgaacc tcctgaatgc ctgagactct agcagaaatg gcctgtttgt 588
 acatttatat ctcttccttc tagttggctg tatttcttac tttatcttca tttttggcac 648
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 gctacttcct acagtatttt ggtcaatatt tggaatgcgt tttagttctt caccttttaa 828
 attatgtcac taaactttgt atgagttcaa ataaatattt gactaaatgt aaaatgtga 887

<210> 4

<211> 88

<212> PRT

<213> Homo sapiens

<400> 4

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 Asn Ile Asn Ile Pro Met Gly Ala Phe Arg Pro Gly Ala Gly Gln Pro
 20 25 30
 Pro Arg Arg Lys Glu Cys Thr Pro Glu Val Glu Glu Gly Val Pro Pro
 35 40 45
 Thr Ser Asp Glu Glu Lys Lys Pro Ile Pro Gly Ala Lys Lys Leu Pro
 50 55 60
 Gly Pro Ala Val Asn Leu Ser Glu Ile Gln Asn Ile Lys Ser Glu Leu
 65 70 75 80
 Lys Tyr Val Pro Lys Ala Glu Gln
 85

<210> 5

<211> 75

<212> PRT

<213> Xenopus laevis

<400> 5

Met Ser Lys Gln Pro Ala Ser Asn Ile Arg Ser Ile Gln Ala Asn Ile
 1 5 10 15
 Asn Ile Pro Met Gly Ala Phe Arg Pro Gly Ala Gly Gln Pro Pro Lys
 20 25 30
 Arg Lys Glu Phe Ser Thr Glu Glu Glu Gln His Val Pro Thr Pro Glu
 35 40 45
 Ser Glu Glu Lys Ser Glu Glu Lys Lys Pro Ile Pro Gly Ala Val Lys
 50 55 60
 Leu Pro Gly Pro Ala Phe Asn Leu Ser Glu Thr
 65 70 75

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 <211> 172
 <212> DNA
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 gcatctggct gcctgggact tcccttaggc agtaaacaaa tacataaagc ag 172

<210> 7
 <211> 57
 <212> DNA
 <213> Homo sapiens

<400> 7
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<210> 8
 <211> 87
 <212> DNA
 <213> Homo sapiens

<400> 8
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 aaagaatgta ctctgaagt ggaggag 87

<210> 9
 <211> 149
 <212> DNA
 <213> Homo sapiens

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 aaagctgaac agtagtagga agaaaaaag 149

<210> 10
 <211> 422
 <212> DNA
 <213> Homo sapiens

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 tatattgtatg atgattgtga acctcctgaa tgcctgagac tctagcagaa atggcctgtt 120
 tgtacattta tatctcttcc ttctagttgg ctgtatttct tactttatct tcatttttgg 180
 cacctcacag aacaaattag cccataaatt caacacctgg aggggtgtgt tttgaggagg 240
 gatatgattt tatggagaat gatatggcaa tgtgcctaac gattttgatg aaaagtttcc 300
 caagctactt cctacagtat tttgggtcaat atttggaaat cgttttagtt cttcaccttt 360
 taaattatgt cactaaactt tgtatgagtt caaataaata tttgactaaa tgtaaaatgt 420
 ga 422

<210> 11
 <211> 40
 <212> PRT
 <213> Patinopecten sp.

<400> 11

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Gln Trp Trp Lys Leu Tyr Ser Lys Val Lys Pro Leu Leu Ser Ile Ala
      20           25           30
Arg Gln Glu Glu Glu Met Lys Glu
      35           40
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<210> 12

<211> 40

<212> PRT

<213> Rattus norvegicus

<400> 12

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Leu Val Ile Gln Trp Asn Ile Arg Ala Phe Met Gly Val Lys Asn Trp
 1           5           10           15
Pro Trp Met Lys Leu Tyr Phe Lys Ile Lys Pro Leu Leu Lys Ser Ala
      20           25           30
Glu Thr Glu Lys Glu Met Ala Asn
      35           40
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<210> 13

<211> 40

<212> PRT

<213> Rattus norvegicus

<400> 13

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Phe Cys Ile Gln Tyr Asn Ile Arg Ala Phe Met Asn Val Lys His Trp
 1           5           10           15
Pro Trp Met Lys Leu Phe Phe Lys Ile Lys Pro Leu Leu Lys Ser Ala
      20           25           30
Glu Thr Glu Lys Glu Met Ala Thr
      35           40
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<210> 14

<211> 40

<212> PRT

<213> Homo sapiens

<400> 14

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Glu Asn Asn Val Met Asn Ile Arg Gln Phe Asn Cys Ser Pro His Pro
 1           5           10           15
Tyr Trp Leu Pro Asn Phe Met Asp Val Phe Thr Trp Ser Leu Pro Phe
      20           25           30
Val Gly Glu Lys Arg Val Thr Glu
      35           40
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<210> 15

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> consensus sequence

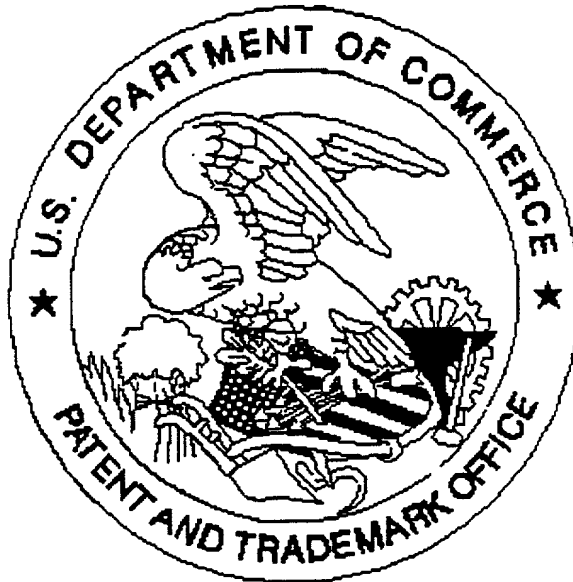
<221> VARIANT

<222> (1)...(6)

00440504004

Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu
1 5 10 15
Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln
20 25

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for scanning. (Document title)

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